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Research Article

Antioxidant and diuretic effects of flower extract of *Laurus nobilis*

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Abstract: Diuretic medications are widely used and can come with negative effects. Because they are effective and have fewer adverse effects than other treatments for renal illness, medicinal plants have become increasingly important. This study aimed to investigate the antioxidant ability and the impact of *Laurus nobilis* extract (flower) on diuresis in rats. Two doses of 200 mg and 400 mg of *Laurus nobilis* extract were used to treat rats for thirty days. Then, we assessed all changes induced in urine and plasma parameters of rats, using furosemide as a standard drug. Further, we evaluated the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant ability (DPPH and FRAP) of the tested extract. The results obtained show that the administration of a single dose of *Laurus nobilis* extract improved the urine flow significantly after 4 h of treatment. Similarly, both doses of the tested extract enhanced sodium, potassium, and chloride excretion without inducing hypokalemia. A similar tendency was recorded for both urine and creatinine, while the results of the furosemide group revealed a significant hypokalemia effect of the standard drug. *Laurus nobilis* demonstrated superior antioxidant and diuretic effects without inducing hypokalemia due to the higher content of phenolic and flavonoid content. However, more advanced studies are required to explore the constituents of *Laurus nobilis* extracts and essential oils, as well as to test their pertinent biological activities.

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Laurus nobilis flowers, Antioxidant activity, Diuretic effect.

1. INTRODUCTION

Natural and sustainable products have gained a reputation in different fields of drug discovery to treat the most devastating diseases, including diabetes, obesity, cancer, and kidney diseases (Soetan, 2008; Hosseinimehr, 2014; Chen *et al.*, 2018; Ojulari *et al.*, 2019). The interest in herbs arose as a result of the negative effects of conventional and chemical medication (Glynn & Bhikha 2019). Kidney diseases are noncommunicable pathologies that have risen in recent years and chronic kidney ailments affect approximately 10% of the adult population around the

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world (Vos *et al.*, 2017). Diuretic drugs adjust blood volume and mineral elements in body fluids. They have a significant effect on the treatment of numerous ailments, such as heart failure, hypertension, kidney failure, and edema (Hymes & Warshaw, 1987; Salvetti & Ghiadoni, 2006). Furosemide is a conventional drug widely used as a diuretic agent that mostly induces a wide range of side effects, including mineral element abnormalities, acid-base disturbance, and glomerular injuries (Eid *et al.*, 2021). Currently, scientists have found a wide variety of bioactive substances in natural and cultivated plant resources that have profound impacts, drawing a lot of interest to investigate their advantageous features.

Medicinal herbs are considered a good source of inexhaustible molecular entities, including bioactive molecules (Unuofin & Lebelo, 2020; Macharia *et al.*, 2022; Soussi *et al.*, 2022). *L. nobilis* is a small perennial tree and belongs to the Lauraceae family (Nava *et al.*, 2021). This plant is characterized by its aromatic and fragrant effects, and it is used traditionally against a wide range of diseases, including rheumatism, dermatitis, digestive system disorders, and eructation (Usmani *et al.*, 2021). For example, areal and ground parts of *L. nobilis* are traditionally used in the eastern Mediterranean basin, such as in Türkiye, as diuretic agents (Patrakar *et al.*, 2012). The abundance and diversity of phytochemical content in *L. nobilis* are suggested to be the principal factors of its various biological effects, including the antioxidant ability (Bourebaba *et al.*, 2021; Dobroslavić *et al.*, 2021), antihyperglycemic (Bourebaba *et al.*, 2021), antimicrobial effect (Fidan *et al.*, 2019), and anti-inflammatory effect (Brinza *et al.*, 2021).

Currently, the investigation of phytochemical constituents in *L.nobilis* using a UPLC–MS/MS approach has detected a large range of bioactive substances, including kaempferol and quercetin glycoside as the predominant bioactive compounds (Dobroslavić *et al.*, 2021). Similarly, phytochemical screening via gas chromatography-mass spectrometry analysis showed significant chemical constituents in the acetonic extracts of *Laurus nobilis* (Batiha *et al.*, 2020). The diversity and complexity of the chemical composition of this plant support its capability to normalize caspase activity through modulation of pro-apoptotic pathways and decreasing the gene expression of inflammatory markers such as c-Jun, NF-KB, and Tlr4 transcripts (Bourebaba *et al.*, 2021). Further, the antioxidant potential of *L. nobilis* was investigated on different occasions. Recently, the antioxidant potential of *L. nobilis* was proven by reducing reactive oxygen species (ROS) via the control of the mitochondrial OXPHOS pathway (Bourebaba *et al.*, 2021). By their ability to neutralize the free radical DPPH, the essential oils of *L. nobilis* also demonstrated significant overall antioxidant properties (Mssillou *et al.*, 2020). The chemical constituents of *Laurus nobilis*, such as kaempferol and quercetin, are effective against nephrotoxicity induced by cadmium and calcium oxalate crystal (Guan *et al.*, 2021; Yuan *et al.*, 2021). Further, the synergetic interaction between different pharmacological active components of *L. nobilis* can be attributed to kidney protection.

In this study, we looked into the antioxidant potential of flowers from the *Laurus nobilis* species that were gathered in Northwest Morocco. In parallel, we sought to assess the diuretic activity of two doses of *Laurus nobilis* flowers and contrasted them with a commonly prescribed standard medication. These findings may offer fresh information on the pharmacological characteristics of native *Laurus nobilis* in Morocco and Northwest Africa as well as open up new research directions for comparative investigations in the future.

2. MATERIAL and METHODS

2.1. Preparation Extract

In this study, we used flowers of wild *Laurus nobilis* to prepare the plant extracts. The plant samples were collected in the vicinity of Moulay Bouslham city, Northwest Morocco, in February 2019. Further, sampled flowers were air-dried and crushed to get a fine powder, which

was utilized to prepare the extract. The extraction procedure was performed using a hydroethanolic solution (70%) with maceration. A ratio of ¼ (solid/liquid) was used, and the obtained extract was filtered before future analysis.

2.2. Total Phenolic and Flavonoid Contents

The evaluation of *Laurus nobilis's* antioxidant capacity was built on the total phenolic content (TPC) and total flavonoid content (TFC) measurements. The phenolic content was ascertained using the colorimetric method that (Singleton *et al.,* 1999) had been previously discussed. In brief, 400 liters of sodium carbonate solution (7.5%) were added after 100 liters of extract had been mixed with 500 liters of Folin-Ciocalteu (0.2 N) and vortexed. After two hours of incubation, the absorbance at 760 nm was measured using a spectrophotometer (Perkin Elmer Lambda 40 UV/VIS) using gallic acid as a reference. The results were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

The steps provided by Kong *et al.,* (2012) were followed in order to evaluate the flavonoid content. In our case, 100 liters of extract were mixed with 150 liters of aluminum trichloride (AlCl3) (20%), 150 liters of sodium nitrite, and 200 liters of sodium solution. After one hour of incubation at room temperature, the mixture's absorbance was determined at 510 nm using a spectrophotometer (Perkin Elmer Lambda 40 UV/VIS). Mg QE/g, or milligrams of quercetin equivalent per gram of extract, was used to show the results.

2.3. Antioxidant Activity

The antioxidant activity of *Laurus nobilis* flower extract was examined using DPPH and FRAP assays. The scavenging effect of *L. nobilis* extract for the DPPH assay was evaluated using the technique defined by Miguel *et al.,* (2014). In our case, the decrease in absorbance was monitored at 517 nm. The following formula was used to determine the percentage of inhibition of the free radical DPPH:

$$\%\text{inhibition} = \left[\frac{A0 - A1}{A0}\right] * 100$$

where A0 refers to the absorbance of the control and A1 refers to the absorbance of the tested sample.

In addition, the inhibition percentage graph of the tested sample was used to determine the IC_{50} DPPH. On the other hand, the FRAP reagent was used to evaluate the capacity of the prepared extract to reduce ferric. In our case, we used the protocol described by Ferreira-Santos *et al.,* (2019), and the FRAP values were expressed as mg/mL.

2.4. Experimental Design

The investigation was conducted with 12 rats weighing between 180 and 200 g in conventional settings (25 °C, 55 % humidity, and 12/12 h (light/dark)). The methods used in the animal house at Sidi Mohamed Ben Abdellah University were the foundation for the manipulation of the animals. Our institutional committee on animal care approved the protocol, which followed the French Technical Specifications for the Production, Care, and Use of Laboratory Animals. Rats were handled and cared for in accordance with the generally acknowledged standards for the use of animals. The animals were then randomly divided into 4 groups of 3 rats each:

- Group 1: served as a control group and was treated with distal water (10 ml/kg b.w);
- Group 2: received furosemide at a dose of (10 mg/kg b.w);
- Group 3: received dose 1 of flower extract (200 mg/kg b.w);
- Group 4: received dose 2 of flower extract (400 mg/kg b.w).

To assess urine flow, urine volume excreted was measured 2, 4, 8, 12, and 24 hours after oral administration of the extract, furosemide, or distal water.

2.5. Biochemical Analysis

All plasma and urine samples from the tested rates were examined for levels of plasma and urinary creatinine, urea, and electrolytes (sodium, potassium, and chloride). On day 30, each rat's blood and urine were taken for various analyses.

2.6. Statistical Analysis

The findings are expressed as the mean ± SD. Graph Pad Prism 5 software was used to perform one-way analysis of variance (ANOVA) followed by Tukey's test. $p<0.05$ was considered significant.

3. RESULTS

3.1. Antioxidants Activity

The results of the phytochemical content and antioxidant capacity of *L.nobilis* flowers are illustrated in Table 1. The total phenol content of the extract was 14.515± 0.79 mg GAE/g, and the value of flavonoid content was 0.73± 0.19 mg QE/g. Concerning the antioxidant activity, the *L. nobilis* extract exhibited high antioxidant potential with IC_{50} DPPH equal to 0.33±0.04 µg/mL and EC50 FRAP equal to 0.67±0.03 mg/mL.

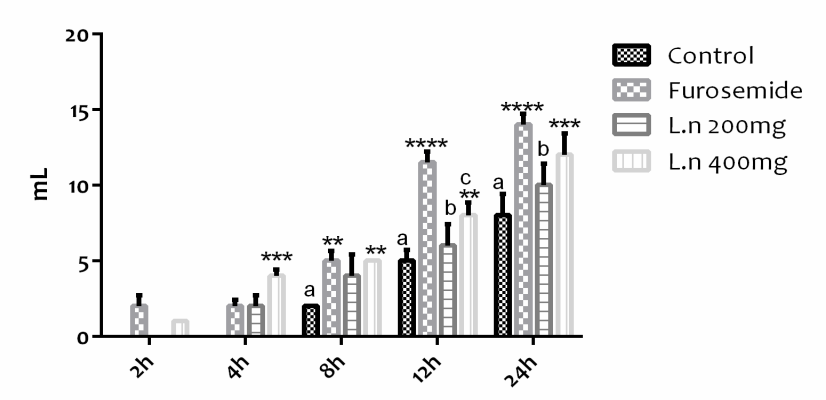
Table 1. Total phenolic and flavonoids contents and antioxidant ability of flower extracts of *L. nobilis.*

	TPC mg GAE/g	TFC mg QE/g	Antioxidant activity	
			IC_{50} DPPH µg/ml	EC_{50} FRAP mg/mL
L. nobilis extract	14.52± 0.79	0.73± 0.19	0.33±0.04	0.67±0.03

3.2. Effect of a Single Dose of *Laurus nobilis* or Furosemide on Urine Volume

The modification of urine flow 24 hours after the administration of both doses of flowers *L. nobilis* extract is presented in Figure 1. The treatment of rats with *L. nobilis* extracts significantly enhanced urine flow after 4 hours of administration. The urine excretion was also increased at each analysis, and the diuresis effect of both doses was significant after 4, 8, 12, and 24 hours ($p<0.05$) compared to the control group (untreated rates). On the other hand, furosemide induced a significant elevation in urine flow compared to the other groups ($p<0.05$).

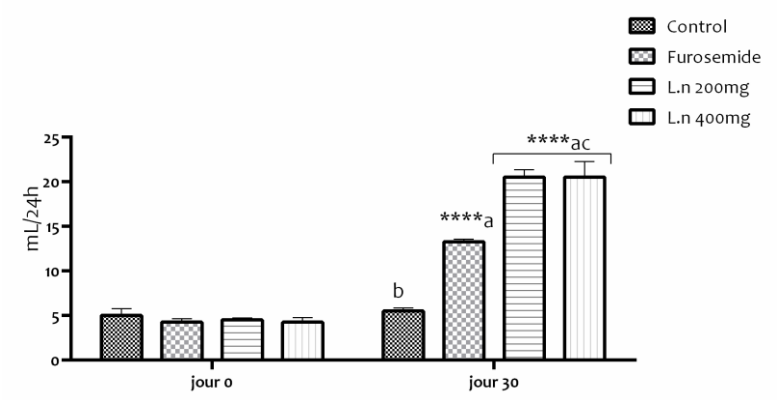
Figure 1. Urine flow of different tested groups during 2 to 24 h of treatment.
(a: Comparison between all groups and the control group;
b: comparison between all groups and the furosemide group; $^{****}<^{**}<^{*}$;
* denote that the value is significantly different from the control with $p<0.05$, $^{**}<0.01$, $^{***}<0.001$).



3.3. Results of Daily Administration of Urine Flow

The effect of *L. nobilis* extract and furosemide on the urine volume of tested rats is presented in Figure 2. The gavage of both doses of *L. nobilis* extracts significantly augmented the urine volume after 30 days of treatment compared to the control group of rats (p<0.05). Equally, the extract of *L. nobilis* significantly augmented the urine flow more than the standard drug (furosemide). The increased rate of urine flow was significantly superior (p<0.05) in the extracts compared to furosemide. In contrast, the obtained results revealed that there were no significant changes between all tested groups on the first day of treatment.

Figure 2. Urine volume in J0 (first day) and J30 (30 days) of treatment.
(a) Comparison between all groups and the control group; (b) comparison between all groups and the furosemide group; (c) comparison between treatments; * denote significant statistic difference and **** denote highly significant difference.



3.4. Impact of Daily Administration of *L. nobilis* Extract on Plasma Parameters

The results of the impact of daily administration of *L. nobilis* extract on plasma urea, creatinine, and electrolytes in tested rats are presented in Table 2. The administration of both doses of *L. nobilis* extract did not induce changes in plasmatic parameters (urea and creatinine) analyzed. Concerning plasmatic electrolytes, furosemide significantly decreased potassium levels after 30 days of treatment. Equally, furosemide induced hypokalemia and presented a significant difference compared to the control group (p<0.05). In contrast, both doses of *L. nobilis* did not induce perturbations of plasmatic electrolytes compared to the control group.

Table 2. Impact of daily administration of two doses of *L. nobilis* extract on plasma urea, creatinine, and electrolytes in normal rats on day 30 ($^{*}p$<0.05 vs control group; #p<0.05 vs the furosemide group).

	Kidney parameters (mg/ml)		Mineral elements (mmol/L)		
Groups	Urea	Creatinine	Sodium	Potassium	Chloride
Control	0.50±0.02	5.00±0.03	164.5±6.40	5.7±0.20#	102.5±5.10
Extract D1	0.43±0.09	5.16±0.22#	170.5±27.5#	5.06±0.44	97.33±1.77*
Extract D2	0.46±0.02	4.80±0.33*	163±15.33	5.1±0.53	107.5±1.50#
Furosemide	0.49±0.02	5.10±0.30	160.2±7.70*	3.9±0.30*	99.75±4.10

3.5. Impact of Daily Administration of *Laurus nobilis* Extract on Urine Parameters

The impact of daily administration of two doses of *Laurus nobilis* extract on urine parameters and electrolytes in rats during 30 days is presented in Table 3. The analysis of recorded results demonstrated that both administered doses of tested extracts significantly increased the elimination of urea and creatinine compared to both the control and furosemide groups (p<0.05). The increased elimination of urea and creatinine was with a dose-dependent effect. The same finding was stated for urine electrolytes. The administration of *L. nobilis* extract significantly boosted the excretion of sodium, potassium, and chloride compared to the standard

drug used (p<0.05). Equally, these results were with a dose-dependent effect. However, the increased excretion did not induce hypokalemia.

Table 3. Impact of daily administration of two doses of *Laurus nobilis* extract on urine urea, creatinine, and electrolytes in rats during 30 days ($^{*}p$<0.05 vs control group. #p<0.05 vs the furosemide group).

	Kidney parameters (mg/dl)		Mineral elements (mEq/dl)		
Groups	Urea	Creatinine	Sodium	Potassium	Chloride
Control	12.89±3.12	47±2.70	108±12.70	62.90±12.70	99±12.00
Extract D1	27.46±1.30*	172.15±28.12*#	122.33±3.77#	392.5±2.50*#	169.33±12.88*#
Extract D2	31±1.33*#	137.80±11.87*#	242±2.66*#	394±4.00*#	276.33±5.11*#
Furosemide	25.76±0.02*	53.1±0.30	147.80±11.90*	160.8±10.9*	198±10.00*

4. DISCUSSION and CONCLUSION

This study revealed new findings on the pharmacological properties and antioxidant potential of wild *L. nobilis* obtained in Northwest Morocco. Our findings assessed the antioxidant potential and determined the amounts of phenolic and flavonoid compounds present in L. nobilis flower extracts. Equally, we evaluated the capacity of *L. nobilis* extracts to improve plasma and urine parameters in rates, which is the first in Morocco and the entire North Africa.

The medicinal plant *L. nobilis* has a number of pharmacological effects (Anzano *et al.*, 2022). In this paper, we studied the antioxidant potential and diuretic effect of the hydroethanolic extract of the wildflowers of *L. nobilis*. The obtained experiments showed that the *L. nobilis* extract demonstrated important antioxidant potential. Moreover, the prepared extracts significantly improved diuretic kidney function with dose-dependent in tested rats. These results were obtained without inducing hypokalemia compared to the control and furosemide drugs (Tables 2 and 3). The standard medication for kidney diseases uses furosemide, which plays a principal role in inhibiting the $Na^{+}/K^{+}/2Cl^{-}$ co-transporter system that enhances urine flow and sodium excretion (Breyer & Jacobson, 1990). Both doses of *L. nobilis* extract enhanced the water, potassium, chloride, and sodium excretion in manipulated rats. In parallel, they prevent the perturbation of plasma electrolyte equilibrium, as shown with the furosemide group (Table 2). Generally, *L. nobilis* is a natural remedy used in traditional medicine and recommended by numerous traditional healers to treat kidney diseases and digestive system disorders (Usmani *et al.*, 2021). It has been demonstrated that extracts of *L. nobilis* show a nephroprotective effect in ant treatment at a level of 300 mg/kg for 9 days without altering kidney histological structure (Taroq *et al.*, 2021). Our results are in agreement with those reported by Taroq *et al.*, (2021). Similar results were also mentioned by Shnewer Mahdi Al-Turfi *et al.*, (2022) in the kidneys of female Wistar rats treated with alcoholic extracts of *L. nobilis*.

The importance of natural products extracted from plants resides in their limited side effects (Tungmunnithum *et al.*, 2018). In our case, treatment of rats with *L. nobilis* extracts did not induce hypokalemia compared to control and furosemide treatments. The beneficial properties of *L. nobilis* were highly associated with its rich chemical composition (Cherrat *et al.*, 2014; Muñiz-Márquez *et al.*, 2014) and predominant phenolic components represented by kaempferol-3-O- hexoside and quercetin-3-Oglucoside (Čulina *et al.*, 2021). In another study, seven compounds, including 1-tricosanol, reynosin, protocatechuic acid, vincetoxicoside B, and vitexin, with strong antioxidant action were detected in *L. nobilis* (Nagah *et al.*, 2021). Therefore, *L. nobilis* exhibits various biological activities, counting antifungal, antiproliferative, cytotoxic, and anti-inflammatory effects (Aourach *et al.*, 2021; Čulina *et al.*, 2021; Olazarán-Santibañez *et al.*, 2021).

Quercetin, as one of phenolic compound of *L. nobilis*, applies an effect on the vascular endothelium, inducing nitric oxide liberation and enhancing vasorelaxation by increasing

kidney filtration (Alarcón-Alonso *et al.*, 2012). Phenolic compounds of *L. nobilis* were implicated in inhibiting Na+/K+ ATPase as a potential way against numerous heart diseases, such as failure and cardiac arrhythmias (Lee *et al.*, 2012). This may be explained by the presence of 0.02±0.001 g EQ/g in the *L. nobilis* extract, and quercetin, as the main component, exerts an effect on the vascular endothelium, enhancing nitrogen monoxide, which is highly associated with diuresis and natriuretic effects (Perez-Rojas *et al.*, 2010). The administration of both doses of *L. nobilis* showed an interesting kind of diuretic ability, interestingly, maintaining the potassium levels in groups treated with the extract studied. In addition, the increase in urinary excretion of potassium and sodium may be due to the considerable content of herbs in mineral salts. The diversity of components of *L. nobilis* and their interaction in vivo could be responsible for the beneficial properties of the studied medicinal plant.

The analysis of the results obtained revealed that the *Laurus nobilis* extract exhibited remarkable antioxidant activity and had a potent diuretic effect after daily administration of two doses in normal rats. Both doses induced significant increases in urine flow and enhanced sodium, potassium, and chloride excretion without affecting serum potassium equilibrium compared to the furosemide group. The findings obtained confirm the utility of *L. nobilis* widely used in traditional medicine and recommended by trade-practitioners.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nor El Houda Tahiri: Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Asmae El Ghouizi**: Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Abderrazak Aboulghazi**: Data curation, Writing – review & editing. **Najoua Soulo**: Data curation, Writing – review & editing. **Badiaa Lyoussi**: Data curation, Writing – review & editing. **Lalla Aicha Lrhorfi**: Data curation, Writing – review & editing.

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Review Article

Different methods of extraction of bioactive compounds and their effect on biological activity: A review

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Abstract: As of yet, there isn't a single technique that is accepted as the standard for extracting bioactive chemicals from plants.

Methods. The effectiveness of both traditional and unconventional extraction methods largely depends on key input variables, knowledge of the composition of plant matter, bioactive chemical chemistry, and scientific knowledge.

Results. The necessity for the most suitable and standardized technology to separate active ingredients for plant matter is highlighted by the utilization of bioactive chemicals in several economic sectors, including the pharmaceutical, food, and chemical industries. This review aimed to discuss there are several extraction methods and their basic mechanisms for the extraction of bioactive substances from medicinal plants.

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1. INTRODUCTION

The choice of the best extraction technique is crucial to both quantitative and qualitative studies of bioactive chemicals derived from plant sources (Smith, 2003; Sasidharan *et al*., 2011). The initial step in every study of a medicinal plant is extraction, which has a substantial impact on the outcome. "Sample preparation techniques" is another name for extraction methods. The majority of the time, this area of research is ignored and carried out by untrained research staff (Azmir *et al*., 2013), despite the fact that sample preparation techniques take up about two-

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thirds of an analytical chemist's time and effort. According to a study (Huie, 2002), the majority of academics think sample preparation is crucial for any analytical research.

The success of bioactive compound analysis is still largely dependent on the methods of extraction, the factors used as input, and the specifics of the plant sections (Moldoveanu & David, 2002). However, it is true that contemporary spectrometric and chromatographic methods have made the process easier than before. Matrix characteristics of the plant component, pressure, temperature, solvent, and time are the most frequent variables influencing extraction operations (Hernández *et al*., 2009). The development of bioactive analysis over the past ten years has been fueled in large part by our growing understanding of the various bioactive compounds' dynamic chemical nature (Dar *et al*., 2015). The pharmaceutical, food additive, and even natural pesticide industries have developed an interest in bioactive compounds derived from natural sources as a result of these enormous technical and technological advancements (Uwineza & Waśkiewicz, 2020). Bioactive substances typically coexist with other substances found in plants. Different plant components, including leaves, stems, flowers, and fruits, can be used to identify and characterize bioactive chemicals. Various extraction techniques can be used to extract plant components. Over the past fifty years, novel techniques that are more ecologically friendly have been developed because they utilize fewer synthetic and organic chemicals, operate more quickly, and provide extracts of higher yield and quality. Ultrasound (Azmir *et al*., 2013), pulsed electric field (Toepfl *et al*., 2006), enzyme digestion (Gaur *et al*., 2007), extrusion (Dunford, 2008), microwave heating (Kaufmann & Christen, 2002), ohmic heating (Lakkakula *et al*., 2004), supercritical fluids (Ghafoor *et al*., 2010), and accelerated solvents (Smith, 2003) have all been investigated as novel strategies to improve the production and selectivity of bioactive components in plant products. Traditional extraction methods like Soxhlet are still used as a benchmark when evaluating the efficacy of newly developed methodologies. There are numerous scientific publications, book chapters, and monographs where non-traditional methods have been thoroughly examined (Wang & Weller, 2006). These works emphasize the application of extraction methods for food additives, nutraceuticals, and many other industries, but they do not discuss the extraction of bioactive chemicals by herbal plants. The goal of the current review is to give a thorough analysis of several methods for extracting bioactive substances from medicinal plants.

2. BIOACTIVE COMPOUNDS

Since the dawn of humankind, plants have been employed by humans. Initially, people just used plants for food, but once their medical capabilities were discovered, this natural flora started to be employed by many different human cultures as a source for disease treatment and health improvement. Through thousands of recipes, Egyptian papyrus demonstrated the value of coriander and castor oil as preservatives, cosmetics, and medicines (Vinatoru, 2001). Hippocrates, Theophrastus, Celsus, Dioscorides, and many other scholars from the Roman and Greek eras reported tens of thousands of medicinal applications for plants (Paulsen, 2010). For a very long time, Romanians have been known for using medicinal herbs. For instance, in his writings from the fifth century B.C., Herodotus wrote that the people who lived north of the Danube River employed the herb Leonurus cardiaca (Motherwort). The Romanian pharmacopeia introduced herbal products in the 19th century, and Cluj city became home to the first institute specialized in medicinal herbs in 1904 (Vinatoru, 2001). The history of bioactive compounds is clearly illustrated by the ancient use of herbal plants. People did not know about bioactive molecules in the past, although there were several applications for these substances in various fields. Typically, secondary metabolites are how plants make their bioactive substances (Bernhoft, 2010). Every living thing, from single-celled bacteria to about a million cellular plants, processes a different set of chemical compounds in order to survive and live. All biological compounds can be separated into two main classes. The first are primary

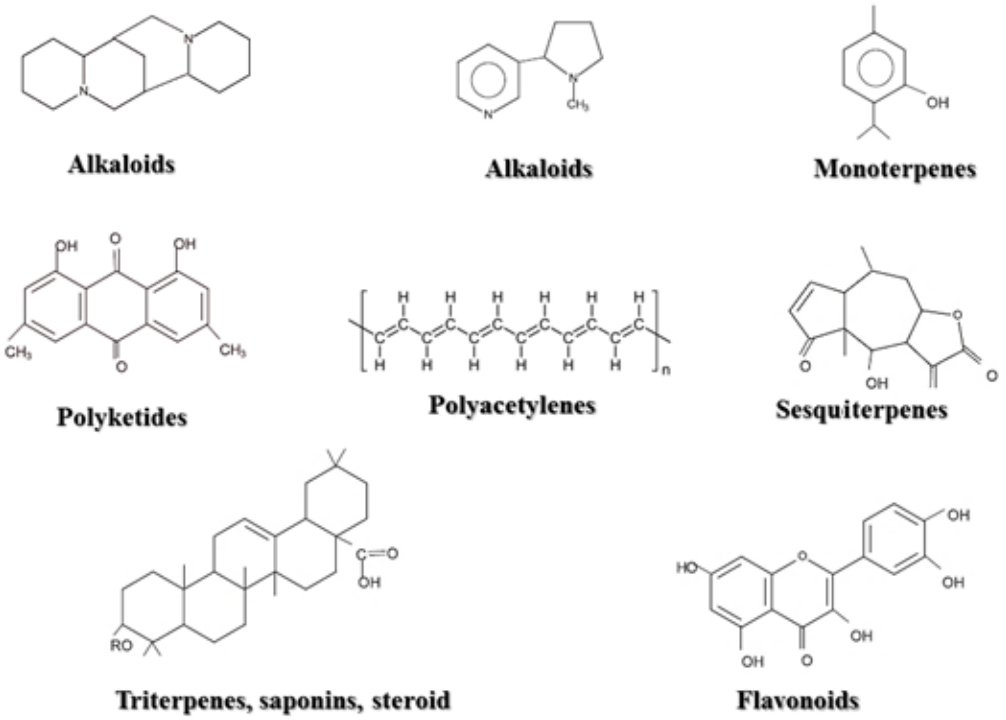
metabolites, which are chemicals that include amino acids, carbohydrates, proteins, and lipids to promote growth and development. Another category is secondary metabolites, a class of chemicals other than primary metabolites that are thought to aid plants in improving their capacity for survival and overcoming local obstacles by enabling them to interact with their environment (Azmir *et al*., 2013). To put it another way, secondary metabolites are metabolites that are regularly created during a growth phase, contain distinctive chemical structures, are typically created as mixes of closely related members of a chemical family, are produced by particular, limited taxonomic groupings of microorganisms, and have no function in growth (although they may have a function in survival) (Erb & Kliebenstein, 2020). Different species primarily choose which secondary metabolites to produce based on their evaluation process and their unique requirements. For instance, flower species provide a scent to entice insects for pollination and fertilization, whereas diseases and herbivores have evolved to produce harmful chemicals to inhibit the growth of nearby plants (Dudareva & Pichersky, 2000). Some of these secondary metabolites are thought to be bioactive because they have an impact on biological systems. The direct definition of plant bioactive compounds is the secondary plant metabolites that have toxic or pharmacological effects on humans and animals (Bernhoft, 2010)

3. SYNTHESIS AND CLASSIFICATION OF BIOACTIVE SUBSTANCES

The classification of bioactive substances into several groups is still arbitrary and depends on the classification's purpose. For instance, pharmacological categorization will not match the scope of categories used in biosynthesis to simplify the description of biosynthetic pathways. According to (Croteau *et al*., 2000), the three major kinds of bioactive chemicals found in plants are (a) terpenoids and terpenes (about 25000 varieties), (b) alkaloids (about 12000 types), and (c) phenolic substances (about 8000 species). Figure 1 provides the general architectur of many kinds of bioactive chemicals.

The bulk of bioactive substances falls into one of several families, each of which has unique structural traits resulting from the manner in which they are constructed in nature. There are four basic mechanisms for the synthesis of bioactive substances, or secondary metabolites: The four processes are presented in order: malonic acid pathway, non-mevalonate (MEP) pathway, shikimic acid pathway, and mevalonic acid pathway (Taiz & Zeiger, 2006). Aromatic amino acids (which derive from the shikimic acid pathway) and aliphatic amino acids both create alkaloids (which are produced by the tricarboxylic acid cycle). Malonic acid and shikimic acid pathways are used to create phenolic chemicals. Terpenes are generated via the MEP and mevalonic acid pathways.

Figure 1. Some types of bioactive chemicals.



4. METHODS USED FOR BIOACTIVE COMPOUND EXTRACTION

It is vital to develop a standardized and comprehensive strategy for screening out these compounds that are beneficial to human health in light of the wide variances between bioactive substances and the variety of plant species (Fabricant & Farnsworth, 2001) described a comprehensive strategy for studying medicinal herbs that began with a name collection of regularly use species and culminated with the industry.

Bioactive chemicals can only be further separated, identified, and characterized before going through the proper extraction procedure. It's essential to utilize multiple extraction methods under different conditions to comprehend the selectivity of extraction from various natural sources. Different methods can be employed to extract bioactive compounds, many of which have remained essentially unchanged for hundreds of years. All of these methods share the following goals: (a) extract specific bioactive substances from complicated plant samples; (b) improve analytical methods' selectivity; (c) raising the concentration of selected compounds will boost the sensitivity of bioassays ; (d) change the bioactive compounds into a form that is better suited for separation, and detection; (e) provide a reliable method that is unaffected by changes in the sample matrix (Smith, 2003).

4.1. Classical and Conventional Methods

The most widely used extraction techniques have relied heavily on liquid-solid extraction for a long time. They typically have simple operations and rely on solvents or heat with various polarities.

4.1.1. *Maceration*

To obtain plant extracts, this procedure involves soaking the plant materials (powered or coarse) in a solvent for a period of two to three days at room temperature while stirring frequently. To prevent solvent evaporation at ambient pressure, an extractor is sealed. In order to liberate the soluble phytoconstituents, the method aims to weaken and break down the plant's cell walls.

After a specific amount of time, the mixture is then squeezed by filtration or decantation (Adegbola *et al*., 2017).

The simplest and most popular method is maceration. The extraction procedure in this stationary method depends on the labor-intensive molecular diffusion concept. Maceration ensures that a new solvent is introduced to the surface of the particles for further extraction and that the concentrated solution that has accumulated on their surface is dispersed (Patel *et al*., 2011).

4.1.2. *Digestion*

In this type of maceration, light heating is used to aid in the extraction process. Menstruum can be used more effectively since the active elements in plant material are not affected by temperature (used as a solvent or combination of solvents for extraction). When the moderately raised temperature is not undesirable, it is employed because it increases the menstrual fluid's ability to dissolve solvents (Zhang *et al*., 2018). Although it can get as high as 50°C, temperatures between 35 and 40°C are the most common. The plant portion that needs to be extracted is put in a container with the liquid that has been preheated to the appropriate temperatures and is kept there while being periodically shaken for a duration that can range from 30 minutes to 24 hours. This method is utilized for herbal material or plant parts that include polyphenolic chemicals or poorly soluble components (Liu *et al*., 2006).

4.1.3. *Infusion*

A simple chemical procedure called infusion is utilized to remove volatile plant material from organic solvents that quickly dissolve or release their active components (Liu *et al*., 2006). Similar to maceration, infusion, and decoction, it entails the process of soaking the plant material in cold or hot water and letting it steep in the liquid. However, the infusion maceration time is shorter. A rotary evaporator can then be used to separate and concentrate the liquid while it is under vacuum.

Infusion is used in the preparation of tea and is recommended for consumption in conditions such as psychophysical asthenia, diarrhea, bronchitis, and asthma, among others. To facilitate urination, lessen irritation, and lower cholesterol accumulation, *Prunus Africana* (pygeum) bark infusion is used orally in tropical Africa (Stéphane *et al*., 2021).

4.1.4. *Lixiviation (Elution)*

The word "lixiviation" (from the Latin lixivium, "lessive") is derived from this concept. Always use a fresh, new solvent that is either cold or hot throughout the extraction process. Water is used as a solvent during the extraction of the components.

4.1.5. *Decoction*

To obtain plant extracts, the present procedure includes boiling the plant material in water. Convection and conduction are two ways that heat is transported, and the kind of substance that can be recovered from plant material depends on the solvent used (Stéphane *et al*., 2021).

The sample is cooked for a predetermined amount of time in a certain amount of water (15 to 60 minutes). After cooling, straining, and filtering, it is provided the desired volume by adding just enough water through the medication. This technique produces more oil-soluble chemicals than maceration and is appropriate for extracting water-soluble compounds from hard plant materials.

4.1.6. *Tincture*

Plant material is extracted using alcohol in this process. Typically, 1:5 ratios of fresh plant material and ethyl alcohol are used. Because they include alcohol, tinctures can be kept at room temperature without going bad (Liu *et al*., 2016).

4.1.7. *Percolation*

It is carried out using dripping the heated solvent through the plant material at a controlled and moderate rate until the extraction is finished before evaporation (for example, 5-7 drops per minute). Usually, the concentrated plant extracts gather near the vessel's bottom. Consecutive filtration can be carried out using filling the percolator with a new solvent and pooling all the extracts to produce a large amount of extract. When producing tinctures and fluid extracts, this process is mostly utilized to extract active ingredients. Its primary drawbacks include the need for huge quantities of solvents, a time-consuming process, and a potential need for experienced personnel (Liu *et al*., 2006).

4.1.8. *Steam distillation and hydrodistillation*

Typically, volatile chemicals, such as essential oil, which are insoluble in water, are extracted from a variety of aromatic and medicinal plants using steam and hydrodistillation techniques. After vapor condensation, the plant products are boiled in water to produce EOs.

Steam distillation takes place at a temperature below the materials' boiling points. The technique works well with thermos-sensitive bioactive substances, such as naturally occurring aromatic compounds. The target compound can then be released from a matrix as a result of the heat causing pore rupture in the sample. According to Raoult's law, the boiling point will decrease when two immiscible liquids are combined. As a result, the mixture's evaporation will approach that of the water in a mixture of volatile chemicals with boiling points range of 150-300°C and water with a boiling temperature of approximately 100°C (Carroll *et al*., 2009; Afroz *et al*., 2015). The fundamentals of steam distillation and hydrodistillation are comparable. In a nutshell, plant material is dissolved in water or a suitable solvent, then heated in the alembic to boiling under air pressure. After liquefying EOs vapors and water in a condenser, the condensate is collected in a decanter, and the EOs are then separated from the water/solvent. Isotropic distillation is the foundation of the extraction theory. The three primary kinds of hydrodistillation are water immersion, direct vapor injection, vapor injection, and hydrodistillation with immersion in water. The amount of time needed for distillation varies on the type of plant material (Afroz *et al*., 2015).

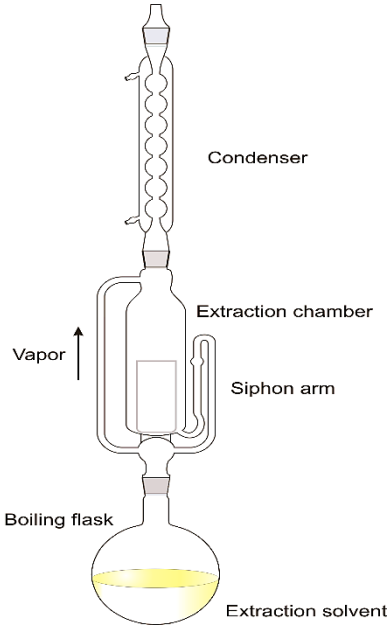
4.1.9. *Soxhlet extraction, continuous hot extraction, or soxhletation*

The Soxhlet apparatus is used in this approach, which involves placing a porous bag or "thimble" containing the finely powdered sample in the thimble chamber. The porous bag is produced from cellulose or sturdy filter paper. Franz von soxhlet created the first soxhlet apparatus in 1879 (Figure 2) (Meyers *et al*., 2004). Warming the extraction solvents in a flask with a flat bottom causes them to evaporate into a sample thimble, which they then condense in a condenser and drip back into. The operation is repeated when the liquid content is drained back into the bottom flask when it reaches the siphon arm (Stéphane *et al*., 2021). The drawbacks include the inability to stir and the need for a significant volume of solvent. Because thermolabile chemicals may be degraded by extended exposure to heat (large extraction times), this approach is not appropriate for them. It is a recognized traditional approach for figuring out how much fat is in various foods (Hemmami, Seghir, *et al*., 2020). The most notable drawbacks of this procedure are exposure to dangerous and combustible liquid organic solvents, and the extremely pure extraction solvents required may increase costs. Additionally, the Soxhlet gadget is unable to provide shaking or stirring to quicken the process (Lee *et al*., 2014).

Comparatively speaking, it uses less solvent than maceration does. Additionally, only one batch of the warm solvent is recycled rather than numerous portions going through the sample (Jha & Sit, 2022; Zeghoud *et al*., 2023). Additional benefits of this method include its straightforward operational mode, its adaptability to higher temperatures that speed up the kinetics process, its low initial investment cost, the lack of filtering, and the solvent's constant

contact with the sample. With heat from the distillation flask, it keeps the extraction temperature comparatively high (Lee *et al*., 2014; Medina-Remón *et al*., 2017; Zhang *et al*., 2018).

Figure 2. Representation of the Soxhlet extraction apparatus.



4.1.10. *Serial exhaustive extraction*

It is a common extraction technique that entails a series of extractions using different solvents that get polarized as they get more polar. The goal is to make it possible to extract chemicals with several ranges of polarities (Stéphane *et al*., 2021).

4.1.11. *Fermentation (aqueous-alcoholic extraction)*

Some pharmaceutical treatments use fermentation as a method of obtaining active ingredients. The crude medication, which can be either a powder or a decoction, is soaked for a predetermined amount of time during the extraction process. After fermentation, alcohol is produced on-site, making it easier to extract the plant material's active ingredients. As a result, alcohol is produced and acts as a preservative. If the fermentation is going to be done in an earthen jar, the water needs to be heated to a boil first. In large-scale manufacturing, porcelain jars, wooden vats, or metal containers are utilized in place of earthen vessels. There isn't a standard for this technique yet (Chhipa & Sisodia, 2019).

For aromatic plants, methods including expression, effleurage (cold fat extraction), and hydrolytic maceration followed by distillation might be used. Some of the most current extraction techniques for aromatic plants include micro distillation, protoplast extraction, solid phase microextraction, and headspace trapping (Stéphane *et al*., 2021).

These strategies are the simplest and most straightforward. Despite the development of more sophisticated extraction techniques, active plant components are still obtained from plants using the potential of traditional solid-liquid extractions. These techniques are criticized because they use a lot of solvents and take a long time to extract, which can kill certain metabolites. The solvents employed in these soaking procedures are essential. There are numerous other cutting-edge extraction techniques have been created (Stéphane *et al*., 2021).

4.2. Innovative (non-conventional) Techniques

The advancement of extraction technology has been advancing steadily in recent years. They are additionally referred to as modern advanced methods.

4.2.1. *Microwave-assisted extraction (MAE)*

Microwaves have wavelengths between 1 cm^{-1} to 1 m^{-1} and operate in the 300 MHz to 300 GHz area of the electromagnetic spectrum of light (Khennouf *et al*., 2003). Two parallel oscillating fields that make up these waves serve as energy and information carriers.

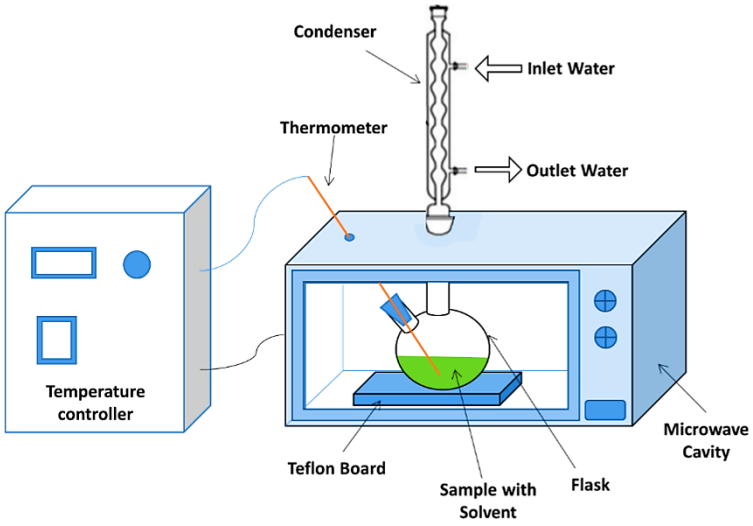
The use of microwave energy speeds up the heating in this extraction procedure. Each molecule is exposed to the microwave field, which has direct impacts such as decreasing temperature gradients, reducing heat-related volume creation, reducing equipment size due to greater process rates, and increasing productivity by making better use of the same equipment's process volume (Gholamhoseinian *et al*., 2012). Given that MAE employs water or alcohol under regulated pressure and elevated temperature, it is a practical green solvent extraction method (Figure 3).

This technique has shown a variety of advantages, including steadiness and ease of handling. According to numerous research, MAE is substantially faster than traditional procedures for extracting active ingredients from plant materials and has greater yields (Adegbola *et al*., 2017; García-Risco *et al*., 2017). MAE can be suggested as a possible replacement for conventional solid-liquid extraction methods. These are a few of the potential benefits: A few milliliters of solvent can be used (need a few milliliters of solvent); The length of extraction might be from seconds to a few min (15 to 20 min); It improves extraction yield; advantageous for thermolabile ingredients; A small plant sample of a few milligrams can be used to extract the trace amounts of heavy metals and pesticide residue; It offers a stirring during extraction, which improves the mass transfer phenomena (Adegbola *et al*., 2017; Nitiéma *et al*., 2018).

Because MAE intensification requires specialized equipment to operate because electricity creates waves, it requires more upfront capital and ongoing operational expenses than traditional approaches (Kumar *et al*., 2010). Banar and colleagues used traditional techniques (maceration, reflux, Soxhlet, hydro distillation, ultrasound- and microwave-assisted extraction (MAE)) with various solvents to extract the bioactive components using Urtica dioica cultivated in Lebanon. According to their findings, MAE was the most successful method. The amount of extracted chemicals was increased while the extraction time was shortened, and less solvent was employed (Sohgaura *et al*., 2018).

When extracting caffeine and polyphenols from green tea leaves for 20 hours at room temperature, MAE achieved a greater extraction yield in 4 minutes (Pan *et al*., 2003). Instead of employing a traditional solvent extraction method for 10 hours, the targeted MAE technique's 15-minute ginsenosides extraction yield from ginseng root was superior (Dhobi *et al*., 2009). Exhibited improved extraction efficiency of MAE when silybinin, a flavonoid from the plant *Silybum marianum*, was extracted instead of more traditional extraction methods, including Soxhlet and maceration.(Jila *et al*., 2011) extracted certain bioactive substances (E-and Z-guggulsterone, tannin, and cinnamaldehyde) under ideal conditions from a variety of plants and demonstrated that MAE is a quicker and simpler procedure than traditional extraction processes. By using MAE, (Chiremba *et al*., 2012). Successfully liberated bound phenolic acids from hard and soft sorghum, as well as maize bran and flour fractions. The solvent concentration, extraction period, and microwave power of the Chinese quince *Chaenomeles sinensis* MAE method were modified to enhance the recovery of phenolics and flavonoids and to improve the extracts' ability to donate electrons (Teng *et al*., 2009).

Figure 3. shows a schematic representation of the microwave-assisted extraction equipment (All rights reserved to (Zghaibi *et al*., 2019)).



4.2.2. *Sonication extraction or ultrasound-assisted extraction (UAE)*

The ultrasound frequencies used in this extraction technique range from 20 to 2000 kHz; this makes cell membranes more permeable and causes cavitation. Despite the process's occasional usefulness, its exorbitant cost prevents it from being used widely. The procedure's primary drawback is the occasionally seen but well-documented harmful impact of ultrasonic radiation on the active ingredients of pharmacological plants, which results in the production of free radicals and, consequently, unfavorable alterations to the drug molecules (Chhipa & Sisodia, 2019). Extraction time, solvent, power, plant material, Liquid/Solid (L/S) ratio, frequency, intensity, and amplitude are all factors that impact how effective UAE is. UAE was superior to other cutting-edge extraction techniques and offered the lowest carbon emissions, greatest mass and heat transmission efficiency, and lowest energy use. According to reports (Iqbal *et al*., 2017), it produced a high level of total phenolic content, antioxidant activity, or particular active chemicals.

Because ultrasonic energy makes it easier for inorganic and organic chemicals to leach by the plant matrix, the main advantage of UAE may be seen in samples of solid plants (Herrera & De Castro, 2005). The most likely process is the enhancement of mass transfer by ultrasound and the rapid access of solvent to plant cell components. The two primary types of physical phenomena involved in the ultrasonic extraction mechanism are (a) Via diffusion mechanism within the cell wall and (b) elution of the contents inside the cell once the walls are broken (Palma & Barroso, 2002). For a successful extraction, the degree of grinding, the sample's level of moisture, solvent, and particle size are all crucial considerations. Additionally, the parameters that control ultrasound action are frequency, pressure, temperature, and period of sonication. Various classical procedures have also been combined with UAE because it is believed that they will heighten the effectiveness of a conventional system. To increase extraction effectiveness, the ultrasonic device in the solvent extraction machine is placed correctly (Hemmami, Ben Seghir, *et al*., 2020).

UAE benefits include a decrease in energy use, extraction time, and solvent usage. Improved blending, quicker energy transfer, decreased thermal gradients, selective extraction, extraction temperature, smaller equipment, quicker reaction to process extraction control, rapid startup, increased output, and the removal of process stages are additional benefits of using ultrasound energy for extraction (Chemat *et al*., 2008).

UAE appears to be a successful extraction method for obtaining bioactive substances from herbal plants (Rostagno *et al*., 2003). Demonstrated the effectiveness of the mix-stirring approach for extracting daidzin, malonyl genistin, and genistin, glycitin, which are four isoflavone derivatives derived from soybean. Depending on the solvent used, researchers discovered that ultrasonic could increase the extraction yield (Herrera & Luque de Castro, 2004). Developed a semi-automatic approach based on ultrasounds to extract phenolic components such as rutin, naringenin, ellagic acid, naringin, quercetin, and strawberry kaempferol using a duty cycle of 0.8 s for the 30s (Li *et al*., 2005) discovered that fresh bark, fresh leaves, and dry bark of Eucommia ulmodies Oliv recovered chlorogenic acid more effectively. By UAE at optimal conditions (20/1 solvent to sample ratio, 70% methanol, and 30 minutes) than conventional extraction procedures (Yang & Zhang, 2008).

4.2.3. *Accelerated solvent extraction or pressurized liquid extraction*

Other names for pressurized liquid extraction include accelerated solvent extraction, pressurized solvent extraction, and improved solvent extraction system (Mukherjee & Patra, 2016). Alternatives to Soxhlet extraction, sonication, maceration, percolation, etc. Dionex Corporation invented pressurized liquid extraction in 1995. It is an automated process that uses a lot of pressures (4 to 12 MPa), high to moderate temperatures (50 to 100 °C) combination with temperatures above their boiling points, and liquid solvents (organic or either aqueous, mixtures, or single) to extract solid materials (Weaver, 2014). When employing water as the extraction solvent, the processes are referred to as hot water extraction, high-temperature water extraction, liquid water extraction, subcritical water extraction, superheated water extraction, and hot water extract pressurized (Mukherjee & Patra, 2016). Solvent, sample size, pressure, pH, temperature, extraction time, and flow rate are all common characteristics that affect the PLE process, with solvent type and temperature being the two most crucial ones *(Barzegar et al*., 2015).

Liquid solvents that are friendly to the environment are employed at moderate to high-pressure levels throughout this extraction process to boost their effectiveness (Sarkar *et al*., 2015). The solubility of the analytes is raised, the matrix-analyte interactions are broken, resulting in a higher diffusion rate, and the extraction process is sped up using raising the solvent's diffusivity. All of these effects are caused by the increased temperature. Contrarily, the increased pressure encourages the solvent to enter the matrix pores while keeping it in a liquid form and preventing it from boiling (Liu *et al*., 2016; Tripathi, 2018).

The key benefits of this method include: (i) a faster extraction time of 15-50 minutes, (ii) the use of fewer solvents (15 - 40 mL), and the absence of filtering. The main drawbacks, however, are expensive equipment and the requirement for continuous variable adjustment to avoid a matrix-dependent efficiency (Sarkar *et al*., 2015). By boosting solubility and mass transfer rates as well as reducing solvent viscosity and surface tension, a higher extraction temperature can help analytes dissolve more readily (Ibañez *et al*., 2012).

PLE was observed to significantly solvent and reduce time consumption use when compared to the conventional soxhlet extraction (Álvarez-Muñoz *et al*., 2004). Today, PLE is being explored as a possible substitute method for supercritical fluid extraction for the extraction of polar chemicals (Kaufmann & Christen, 2002). The extraction of organic contaminants by environmental matrices that are stable at high temperatures is another use for PLE (Wang & Weller, 2006). The extraction of bioactive chemicals by marine sponges has also been done using PLE (Ibañez *et al*., 2012). Research regularly contains applications of the PLE approach for acquiring natural products (Kaufmann & Christen, 2002). Additionally, PLE is widely considered a green extraction process due to the small amount of organic solvent required (Ibañez *et al*., 2012). The use of PLE to extract bioactive substances from various plant sources

has been shown to be successful. Isoflavones were successfully recovered from freeze-dried soybeans using optimal conditions (Shen & Shao, 2005).

Contrasted ASE, Soxhlet extraction, and ultrasonically assisted extraction for the extraction of sterols from tobacco. Due to its quicker process and less solvent use, PLE has been thought of as an alternative to conventional techniques in terms of yield, solvent consumption, and extraction time repeatability. Water solvent at 50-130 °C The ethanol and water mixture (70/30) was weakly effective as a solvent for the extraction of flavonoids from spinach (Howard & Pandjaitan, 2008; Luthria, 2008) revealed that the PLE extraction of phenolic chemicals from parsley (Petroselinum crispum) flakes is affected by solid to solvent ratio factors, static time, pressure, particle size, flush volume, and temperature. Galanthamine and lycorine (Amaryllidaceae alkaloids) were extracted using Narcissus jonquilla using an optimized PLE process, which was more successful than hot-solvent extraction, UAE, and MAE (Mroczek & Mazurek, 2009).

4.2.4. *Supercritical fluid extraction (SFE)*

Supercritical fluids are employed as the extracting solvent in SFE to separate components from the matrix (Ahmad *et al*., 2019).The fluid for extraction that uses CO_2 has various benefits. Additional issues include its lower boiling point (31°C) and critical pressure. Additionally, carbon dioxide is cheap, safe, and abundant in nature. However, while being the favored fluid for SFE, carbon dioxide has a number of polarity restrictions. Solvent polarity is important for extracting polar solutes and when there are significant analyte matrix interactions.

Environmental samples, pesticides, food and perfumes, polymers, essential oils, and natural items all find wide use for SFE (Chhipa & Sisodia, 2019).

The essential oil of rosemary (Rosmarinus officinalis) was extracted using S-CO_2 extraction, steam distillation, and hydro distillation by Conde-Hernández and associates. They discovered that SFC extract had better essential oil yields and antioxidant activity than the other two techniques (Pandey & Madhuri, 2010).

Donn *et al*., (2022) used SFE at 313–343 K temperature and pressure ranging from 14–24 MPa to extract the purine alkaloids (theobromine, caffeine, and theophylline) from Ilex paraguaryensis. At 58.6 °C and 9.5 MPa (Giannuzzo *et al*., 2003), The flavonoid naringin, which is extracted from citrus paradise using ethanol (15 wt.%) with supercritical CO_2 modified, yielded higher yields than pure supercritical CO_2. Grape seeds were used to extract procyanidins and polyphenols using SFE, and more than 79% of the catechin and epicatechin from the seeds were released when methanol-modified CO_2 (40%) (Verma *et al*., 2008) Catharanthus roseus leaves were extracted using SFE-under optimal conditions, and the highest catharanthine recoveries occurred at 25 MPa, 80°C, with 6.6 % methanol acting as a modifier for 40 minutes.

4.2.5. *Pulsed electric field (PEF) extraction*

A method known as pulsed electric field extraction involves exposing vegetable matrix to an electrical potential. An electric pulse produced by a transformer raises voltages from 140 or 220 V to 1000 V or more. This high voltage is transformed in a sealed space with metallic electrodes by a capacitor (Bast *et al*., 2014). The cell degradation and the extraction of components from the intracellular vacuoles are both prevented by this "cold" extraction facilitated by PEF (Jamshidi & Cohen, 2017). Because it may boost mass transfer using breaking membrane structures during the extraction technique, it significantly increases the yield and cuts down on time.

Among the variables that can affect the treatment effectiveness of the PEF extraction are the particular energy input, field strength, and heat treatment.It is referred to as a non-thermal approach that lessens the degradation of the components that are thermolabile (Sy *et al*., 2005).

4.2.6. *Enzyme-assisted extraction (EAE)*

A specialized hydrolyzing enzyme is added throughout the extraction process to perform the EAE, an enzymatic pre-treatment. Micelles are generated in the cell wall structure and cell membrane using macromolecules, including polysaccharides and proteins. The primary obstacles to extracting natural products are high-temperature protein coagulation and denaturation during extraction. Due to the enzymes on the cell wall's hydrolytic activity, membrane, and internal macromolecules, this makes it easier for natural compounds to release, and EAE increases the extraction efficiency. In EAE, hydrolyzing enzymes like cellulose, -amylase, and pectinase are frequently used (Tzanova *et al*., 2020). This method is effective for removing a variety of bioactive materials using plant matrices, although, after filtration, the resulting fraction is abundant in tiny water-soluble compounds, including flavonoids and polyphenols (Tzanova *et al*., 2020).

The release of bound molecules and an improvement in total yield have both been attributed to enzymatic pre-treatment, which has been deemed both unique and efficient (Ghandahari Yazdi *et al*., 2019). By dissolving the cell wall and lipid bodies, the structural polysaccharides are hydrolyzed, adding some enzymes to increase recovery during extraction (Łubek-Nguyen *et al*., 2022). Examples of these enzymes are cellulase, a-amylase, and pectinase. Either enzyme-assisted cold pressing (EACP) or enzyme-assisted aqueous extraction (EAAE) are two methods of enzyme-assisted extraction (Latif & Anwar, 2009). EAAE techniques have typically been developed primarily for the extraction of oils from various t seeds (Sharma *et al*., 2002). Since the EACP system does not have access to polysaccharide-protein colloid, as is the case in EAAE, enzymes are employed to hydrolyze the seed cell wall in this method (Concha *et al*., 2004). It is understood that a number of variables and important factors for extraction include the kind and concentration of the enzymes, plant material particle size, the ratio of solid to water, and the length of the hydrolysis process. According to (Niranjan & Hanmoungjai, 2004), plant materials' moisture content has a significant role in the enzymatic hydrolysis process. Because it is nontoxic and nonflammable (Azmir *et al*., 2013) characterized EACP as a perfect alternative for extracting bioactive components from oilseed, it was discovered that oil extracted using enzyme-assisted techniques included more free fatty acids and phosphate than oil extracted using conventional hexane methods (Azmir *et al*., 2013). Because it substitutes water for organic chemicals as the solvent in the extraction of bioactive substances and oil, the EAE is acknowledged as an environmentally beneficial method (Puri *et al*., 2012) EAE of phenolic antioxidants from grape pomace was examined during the manufacturing of wine by (Meini *et al*., 2019) who discovered a link between the yield of total phenols and the degree of enzyme-mediated breakdown of plant cell walls. (Landbo & Meyer, 2001) found that utilizing different enzymes increased the release of phenolic chemicals from Ribes nigrum pomace. The recovery was maximum with celluzyme MX when (Li *et al*., 2006) EAAE was used to determine the total phenolic content of five citrus peels (Yen Ben lemon, grapefruit orange, mandarin, and Meyer lemon). Another important study conclusion was that higher enzyme concentration significantly improved phenolic antioxidant extraction.

4.2.7. *Turbo-distillation extraction or turbo-extraction* (thrombolysis)

Martel developed turbo-distillation in 1983, and it has been utilized commercially by a number of businesses to extract EOs from tough matrixes (like bark, wood, and seeds). Turbo-extraction, also known as based on extraction, such as turbolysis during agitation and concomitant particle-size reduction. High shearing forces cause cells to rupture, which causes

the active ingredients to dissolve quickly. The plant material is almost entirely depleted and the extraction process takes minutes to finish. Turbo-distillation minimizes extraction time and energy consumption in comparison to hydrodistillation and reduces the degradation of volatile elements (Puri, 2002).

Martins *et al*., (2017) investigated the turbo-extraction of rebaudioside stevioside and stevioside from dried and powdered leaves of *Stevia rebaudiana*. Applying a fractional factorial design to the extraction process allowed researchers to assess the main factors affecting the yield of these glycosides, including the size of the drug powder, the weight ratio of the solvent to the drug, temperature, time, and stirring. Their research showed that turbo-extraction offered hope for the extraction of Stevia rebaudiana glycosides.It sparked a fresh investigation into the extraction of these extracts, which turned into a lucrative industry for emerging nations like Brazil and India (Prakash & Gupta, 2005).

The essential oil obtained using turbodistillation in 30 minutes was demonstrated by Perino and associates to be qualitatively (aromatic profile), and quantitatively (kinetics profile and yield) comparable to that obtained by traditional hydrodistillation in 3 h. They came to the conclusion that this method, which resulted in a shorter extraction time, was ideal for extracting hard matrixes (Puri, 2002). Compared to dynamic maceration, it may be favorable.

4.2.8. *Counter-current extraction* (CCE)

In this procedure, a fine slurry is created from the raw material, which is moist. The target material is delivered just one way within a cylindrical extractor (often as a fine slurry), where it comes into contact with the extracting solvent. Additionally, the initial component shifts, producing a more concentrated extract. In other words, full extraction is feasible when the material quantities and solvent flow rate are adjusted. When high temperatures are used, the procedure is exceedingly effective, quick, and risk-free. Finally, the extracts exit the extractor suitably concentrated at one end, while the residue exits at the opposite end (Chhipa & Sisodia, 2019). His extraction method has many benefits, including A unit amount of the plant material may be extracted using a lot less solvent than with other techniques, including percolation, decoction, and maceration; Since CCE is typically carried out at 25°C, the thermolabile ingredients are not exposed to heat as they would be in most other procedures; Since the medication is crushed in moist circumstances, water dissipates the heat produced during comminution. By doing this, components' thermal damage due to heat exposure is once again prevented; CCE is recognized as more effective and efficient than continuous heat extraction.

4.2.9. *Solid-phase extraction*

In order to chemically separate the various components using the sample preparation method known as solid-phase extraction (SPE) uses solid particles frequently found in cartridge-type devices as chromatographic packing material. Nearly often, samples are in a liquid form (even if certain samples can be used for unique purposes in the gas phase). This technique allows for the separation of dissolved or suspended compounds from other compounds in a liquid mixture based on their physical and chemical properties. Since the chromatographic particles are solid while the sample is liquid, the proper term for this process is "Liquid-Solid Phase Extraction" (Cohen, 2014).

SPE offers several advantages, but four crucial advantages demand special attention: chemical purification and the simplification of complicated sample matrices; minimizing ion enhancement or suppression in MS applications; capacity to fractionate sample matrices for compound class analysis; and traces of extremely low-level chemical concentrations. The solute molecules are preferentially linked to several types of cartridges and disks with varied sorbents in this rapid, economical, and sensitive technique.

4.2.10. *High-voltage-assisted extraction*

With the exception of the electrical discharge occurring through a tiny point, the operation of this equipment operates on a similar concept as PEF. For this, a ground electrode plate is converted into a release from a needle electrode.

In terms of excellent yields, a lot of selectivities, reduced solvent usage, and accelerated extraction times, these so-called greener approaches frequently outperform conventional ones. They are also discovered to be environmentally friendly because they utilize less organic solvents and energy. The literature (Biswas & Biswas, 2005; Vaidya, 2011; Bhateja & Arora, 2012; Lyons *et al*., 2018) describes the combination of extraction techniques to produce high yields overall or high-purity extracts. Its primary benefit is continuous mode operability; this, from an industrial and commercial standpoint, is essential (Bast *et al*., 2014).

4.2.11. *Phytonics process*

In contrast to conventional methods, a novel solvent based on hydrofluorocarbon-134a and a new technique to enhance its exceptional capabilities in the extraction of plant matter provide considerable environmental benefits as well as safety and health benefits. Advanced Phytonics Limited created and patented the "phytonics process" technology (Manchester, UK). The materials typically extracted by this procedure include fragrant elements of EOs and phytopharmacological or biological extracts that may be employed directly without the need for further chemical or physical treatment. The extraction of plant material has been done using the characteristics of the new generation of fluorocarbon solvents. 1,1,2,2-tetrafluoroethane, also known as hydrofluorocarbon-134a (HFC-134a), is the main component of the solvent and has a boiling point of -25°C and an ambient temperature vapor pressure of 5.6 bar. It is non-toxic and flammable. More significantly, this chemical was created to replace chlorofluorocarbons since it does not harm the ozone layer. Using most measures, this is a subpar solvent that cannot dissolve plant waste. The ability to modify the solvents makes the method beneficial since it may be designed to be very selective in extracting a particular class of phytoconstituents by employing modified solvents with HFC-134a. Likewise, different modified solvents may be used to extract a wider range of components. This technique produces biological products with very little residual solvent residuals are consistently less than 20 parts per billion and below detection thresholds. Therefore, the selected solvents have a limited potential for interacting with plant matter and be non-acidic and non-alkaline. The processing facility is sealed at the conclusion of each manufacturing cycle to ensure ongoing recycling and complete recovery of solvents. These devices only operate with electricity, and even then, they use relatively little energy. There is no room for solvents to escape, and even if they did, those that did not include chlorine would not constitute a hazard to the ozone layer. Dry and "eco-friendly" to handle, the biomass that these plants produce as trash.

The phytonics technique is frequently used in biotechnology to extract ingredients for food, beverages, flavored oils, and pharmaceuticals (for example for the synthesis of antibiotics). Producing superior pharmaceutical-grade extracts, pharmacologically active intermediates, phytopharmaceuticals, and antimicrobial extracts are only a few applications for it. Its usage in all of these domains, nevertheless, precludes its application in other areas. From various kinds of plant material, the method is being utilized to extract premium essential oils, aromatic oils, oleoresins, tastes, and natural food colors. The method is also used to refine raw materials that come from other extraction procedures. It offers extraction free of impurities like wax. Numerous biocides are removed from polluted biomass with its assistance (Chhipa & Sisodia, 2019).

4.3. Liquid–Liquid Extraction (Partitioning)

The most frequent next step after the solids have been extracted and the desired organics have been released into the extraction solvent is a liquid-liquid extraction, which takes the positive of combining two (or, sometimes, three or more, which can create two periods) non-miscible solvents, like ether, and water. Polar chemicals should be dissolved in polar solvents, according to the general rule (For instance, proteins, carbohydrates, and amino acids all persist in water). On the other hand, the nonpolar elements frequently stay in the organic phase (Examples include extracting steroids, waxes, terpenoids, and carotenoids into a solvent like ethyl acetate).

When extracting plant material using traditional or cutting-edge techniques, it's critical to reduce interference from substances that might coextract with the target molecules. Additionally, it is necessary to prevent extract contamination, the decomposition of important metabolites, or the formation of artifacts as a result of the extraction process or solvent impurities (Guaadaoui *et al*., 2014); the extracted solution must be filtered to get rid of any particulates, regardless of the extraction technique utilized. Plant extract shouldn't be kept in the solvent for an extended period of time at 25°C or in direct sunlight because of the increased danger of artifact creation and the breakdown or isomerization of extract components that go along with it (Guaadaoui *et al*., 2014).

4.4. Extraction of Specific Metabolites

Before doing additional chromatographic analysis, the chemical research profile of a plant extract and fractionation of a crude extract is useful for separating the principal classes of components from one another. One method based on variable polarity may be used in a plant that produces alkaloids. From plant to plant, the kind and quantity of components that must be divided into various fractions will differ. When labile chemicals are being examined, this approach might be changed (Sy *et al*., 2005).

4.4.1. *Extraction of essential oils* (EOs)

All plant organs generate essential oils; they have a strong perfume and are concentrated aromatic hydrophobic greasy volatile liquids (Gülçin *et al*., 2004). In order to extract them from raw materials, a number of extraction techniques are used, including hydro diffusion, Soxhlet extraction, cold pressing, pressure-assisted expression, or solvent extraction, often known as the scarification technique, gravity, microwave-assisted extraction, microwave hydro diffusion, and water or steam distillation extractions. The easiest extraction method to utilize will rely on how easily the target components will evaporate (volatility) and whether they will be hydrophilic or hydrophobic (polarity) (Liu *et al*., 2002; Agarwal & Rangari, 2003; Howes *et al*., 2003; Triveni *et al*., 2012; Wani *et al*., 2012). However, Soxhlet, hydro distillation, and SFE are the three methods used to extract Eos most commonly applied (Francis *et al*., 2002). The chemical makeup of EOs is considerably influenced by the extraction technique used (Gülçin *et al*., 2004). According to a recent study by Benmoussa *et al*., To improve both the quality and the quantity of the Eos extracted from medicinal and fragrant plants, microwave hydro diffusion and gravity appear to be a quick procedure, a green technology, and a sought alternative technique (Triveni *et al*., 2012).

4.4.2. *Extracting oils and fats*

A large class of non-polar molecules known as lipids are soluble in an organic solvent, for example, diethyl ether, n-hexane, alcohol, or chloroform but are only weakly or totally soluble in water (Kalidas & Mohan, 2010). In contrast to oil, which is a triglyceride that is a liquid or transparent liquid at room temperature, room temperature, triglycerides that make up fats are solid or semi-solid. The degree of solubility, however, determines their chemistry. Vegetable, animal, and marine sources of fats and oils are all possible (Shrinet *et al*., 2021). Pre-treatment

is the first step in a series of procedures needed to produce oilseeds and fats. Before extracting the oil using solvents, it is frequently required to dry, as several organic solvents are not water-miscible and do not readily penetrate the matrix; extraction is ineffective (Hewavitharana *et al*., 2020). Several different types of non-polar molecules, known as lipids, are Because of the makeup of the matrix. Processing techniques are typically neither lipid-specific nor 100% effective at recovering lipid particles. Because they are comparatively non-polar and can thus extract the majority of nonpolar components from crude fat, petroleum ether and diethyl ether are preferred solvents in this situation (Kalidas & Mohan, 2010).

The extraction of edible oils can preserve tocopherols, stop chemical changes in the triacylglycerol, and have adverse effects on taste, appearance, stability, or dietary value. Various traditional and cutting-edge methods, like hot water extraction, microwave-assisted extraction, solvent extraction, cold pressing, supercritical fluid extraction, and high-pressure solvent extraction can be used to extract fats and oils from plants (Shrinet *et al*., 2021). Leaching, washing, diffusion, and dialysis are only a few of the methods used in the extraction of oil (Kalidas & Mohan, 2010). Crude oil is produced from palm oil (seeds of Elaeis oleifera) after being digested and then a pressing stage. In order to release the palm oil from the fruit, digestion aids in the rupture or breakdown of the oil-bearing cells (Gholap & Kar, 2003). Enzyme-assisted extraction is an effective way to increase lipid extraction from a variety of biomasses, including microalgae, soybean, and sunflower (Hegde *et al*., 2014). The three primary adverse effects associated with oil processing are the creation of trans fatty acids, (ii) cis-trans isomerization, and (iii) physical loss (Shrinet *et al*., 2021). To avoid fungus growth and the formation of lipase, which would increase the amount of free fatty acids, oilseeds must not have moisture levels over a particular level prior to processing (Kalidas & Mohan, 2010).

4.4.3. *Volatile organic compounds*

Aromatic chemicals called volatile organic compounds (VOCs) are released by plant tissues. A wide range of VOCs can be produced by plants. They are in charge of giving some dried plants, such as *Camellia sinensis* tea, its distinctive scent. VOCs can consequently be utilized to determine the quality of tea (Shiny *et al*., 2013). As a natural defense against disease and arthropod assaults, many VOCs are released (George *et al*., 2007). In order to extract VOCs, a variety of processes are used, like microwave-assisted hydro-distillation, hydro-distillation, steam distillation, simultaneous distillation solvent extraction, supercritical fluid extraction, solid phase microextraction, purge, and trap (Mallikharjuna *et al*., 2007).

Verde and partners carried out research to define the volatile components and maximize the MAE of the volatile oil terpenes using *Pterodon emarginatus* fruits. According to their study, MAE might be used to extract volatile oils from plants without the use of organic solvents. They demonstrated that even a small amount of water might produce results in extraction. This environmentally friendly technology seems to be a great substitute for extracting terpenes from fragrant plants (Stéphane *et al*., 2021).

All of these methods have advantages and disadvantages that we mention in Table 1.

Table 1. summarizes the pros and cons of the most important extraction methods (Bitwell *et al*., 2023; Rasul, 2018).

Extraction methods	Advantages	Disadvantages
Maceration	- simple process employing straightforward tools and equipment. - No skilled operator is necessary. - process for saving energy. - Ideal simply requires lengthy contact with solvent. - Method that works well for inexpensive, less powerful medications.	- The medication wasn't completely extracted. - It takes a long time and moves quite slowly.
Digestion	- A technique that involves a little warmth during the extraction process and is comparable to maceration. - To prevent the bioactive phytochemicals in the provided plant material from changing due to temperature.	- As a result of heat, the extraction solvent is used more effectively. - To begin the extraction process, the necessary plant parts are added to a container containing the right solvent that has been preheated to the specified temperatures.
Decoction	- Effective for extracting chemicals that are heat stable. - This procedure doesn't call for more complex or expensive tools. - It is simple to carry out.	- The extraction of heat-sensitive compounds is not recommended.
Percolation and Infusion	- Less time-consuming than maceration. - It may be able to extract elements that are thermolabile. - Appropriate technique for expensive and strong medications. - Quicker and more thorough extraction.	- Takes longer than soxhalation. - Additional solvent is needed. - A skilled individual is needed. - Throughout the procedure, particular attention should be paid to the material's particle size.
Steam distillation and hydrodistillation	- Increased oil yield. - The volatile oil's constituents are less prone to hydrolysis and polymerization. - The consistency of the oil quality obtained by steam and water distillation is higher. - This procedure is economical and environmentally benign because no organic solvent is required.	Complete extraction cannot be accomplished. - Some essential oil compounds may hydrolyze as a result of hot water's prolonged action. - It is challenging to manage the temperature, which could cause fluctuating distillation rates. - The method is not profitable.
Soxhlet	- Significant quantities of plant materials can be harvested simultaneously. - Able to use solvent repeatedly - This technique does not call for filtration following extraction. It is a really straightforward procedure. - The repeated interaction of a new solvent with the solid matrix to shift the transfer equilibrium.	- Because the samples are heated to a high temperature for a considerable amount of time, there is a chance that certain compounds will be thermally destroyed. - The extraction procedure requires a lot of labour and takes a long time. - Only a few variables can be manipulated by the process.

5. METHODS OF PLANT BIOACTIVE MOLECULE ISOLATION AND PURIFICATION

Recently, new developments have been made in the separation and purification of plant-based bioactive substances (Brusotti *et al*., 2014). This innovative method enables a comparison between the availability and development availability of various complex bioassays and the availability of precise separation, isolation, and purification methods. Finding a technique that is suitable for screening the source material for bioactivity, such as antibacterial, antioxidant,

or cytotoxicity, while combining simplicity, specificity, and speed, is the aim when looking for bioactive substances (Altemimi *et al*., 2017).

Because animal tests are more costly, time-consuming, and subject to ethical debates, in vitro procedures are typically preferred over in vivo trials. Finding definitive methodologies or protocols to extract and identify certain bioactive materials is difficult due to a number of variables.

This could be because a plant has several components (tissues), many of which create very different compounds, and because the bioactive phytochemicals have a variety of chemical structures and physicochemical characteristics (Kumar *et al*., 2021). The selection and collection of plant materials are regarded as the first steps in the process of isolating and identifying a bioactive phytochemical. The last stage is retrieving ethnobotanical data to identify potential bioactive compounds. The active substances that are responsible for the bioactivity can subsequently be isolated and purified by creating extracts using a variety of solvents.

Techniques for column chromatography can be utilized to separate and purify the bioactive components. The purification of the bioactive molecule is sped up by modern technologies like High-Pressure Liquid Chromatography. The purified chemicals may be recognized using a variety of spectroscopic methods, including UV-visible, mass spectroscopy, Nuclear Magnetic Resonance (NMR), and Infrared (IR) (Manthey & Guthrie, 2002).

5.1. Purification of the Bioactive Molecule

The use of column chromatography and paper thin-layer techniques has allowed for the isolation and purification of several bioactive materials due to their availability in a variety of fixed phases, affordability, and ease, column chromatography and thin-layer chromatography (TLC) are still frequently used(Bajpai *et al*., 2016).

For separating the phytochemicals, alumina, silica, polyamide, and cellulose are the most valuable materials. High concentrations of complex phytochemicals found in plant materials make good separation challenging (Altemimi *et al*., 2017). Therefore, for very valuable separationsit is advantageous to increase polarity utilizing many mobile phases. The fractions of compounds using column chromatography have traditionally been analyzed using thin-layer chromatography. Some analytical methods have been utilized for the separation of bioactive material, such as TLC and silica gel column chromatography (Annadurai, 2021).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The constant need to extract plant bioactive components stimulates research for convenient extraction methods. The creation of the majority of non-conventional extraction procedures is largely due to the growth of chromatography advancement and awareness about the environment. However, since most of these approaches are based on diverse mechanisms and extraction improvement is the consequence of several processes, knowing every component of the non-conventional extraction process is essential, and It is important to look into the incorporation and development of hybrid approaches while taking into account the properties of the plant material and compounds selection. Some of the current approaches still don't have enough experimental data. The assessment of extraction efficiency is influenced by the proper use of standard methods. However, the rising economic significance of bioactive compounds and the commodities that include these substances may encourage the development of more advanced extraction techniques in the future.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Imane Ghenabzia: Conceptualization, Data curation, Writing-Original draft preparation, Writing- Reviewing and Editing. **Hadia Hemmami**: Writing Reviewing and Editing, Visualization, Supervision. **Ilham Ben Amor**: Data curation, Writing-Original draft preparation, Writing- Reviewing and Editing. **Soumeia Zeghoud**: Data curation, Writing-Original draft preparation, Writing, Reviewing and Editing. **Bachir Ben Seghir**: Data curation, Writing-Original draft preparation, Writing- Reviewing and Editing. **Rokaia Hammoudi**: Data curation

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Research Article

Characterization and antibacterial activity of alkaloids and polyphenols extracts from *Haplophyllum tuberculatum* (Forssk.) A. Juss.

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Abstract: *Haplophyllum tuberculatum* is a medicinal plant belonging to the Rutaceae family, is renowned for its various therapeutic properties. This study aims to characterize the alkaloids and polyphenol extracts from this plant and assess their antibacterial activity. Herein, the extraction of polyphenols and alkaloids from this plant was performed by the maceration-method. Folin Ciacalteu's method was used to estimate the total phenolic content, and the qualitative characterization of the two extracts was performed by thin-layer chromatography. Whilst, the antibacterial activity of the two extracts was tested with the disk diffusion method on a solid medium and the minimal inhibitory concentration (MIC) of susceptible bacteria was determined using the agar dilution method. Our results indicate respective yields of 8.39% in polyphenols and 0.37% in alkaloid extracts, while the total phenolic content was estimated to be 74.45 mg GAE/g of dry matter in polyphenolic extract. Thin-layer chromatography analysis allowed choosing the system toluene-acetate-ethanol-concentrated ammonia (40:4:8:3, v/v) to separate *H. tuberculatum* alkaloids, and ethyl acetate-methanol-water (100:13.5:10, v/v/) for the separation of its polyphenols. The same analysis detected traces of quercitin, catechin, and rutin in the polyphenolic extract. Our findings demonstrated good antibacterial activity on Gram-positive strains such as *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, and moderate activity on *Pseudomonas aeruginosa* ATCC 27953, with MICs ranging from 0.625 to 10 mg/mL for alkaloids and from 5 to 20 mg/mL for polyphenols.

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1. INTRODUCTION

The study of plant chemistry continues to be of enduring importance, despite its historical origins. This enduring relevance arises from the fact that the plant kingdom serves as a significant source of a wide array of bioactive molecules that find diverse applications in various industries, such as food production, cosmetics, and pharmaceuticals. Among these compounds, alkaloids and polyphenols play particularly prominent roles (Ferrari, 2002).

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Alkaloids are nitrogenous organic substances, most often of plant origin. They have multiple pharmacological activities and are used as depressants, antispasmodics, vasodilators, and anticancer drugs, but some of them are also used as antimicrobial agents (nicotine, caffeine, morphine, lupine) (Iserin, 2001; Catier & Roux, 2007). However, phenolic compounds constitute a large heterogeneous group of secondary metabolites of plant origin (Sarni-Manchado *et al.*, 2006). The basic structural element is a benzene ring to which one or more hydroxyl groups are linked, free, or committed to another chemical function (ester, heteroside, ether) (Bruneton, 1999). Research on phenolic compounds is very advanced because of their various physiological properties such as anti-allergic, anti-artherogenic, anti-inflammatory, hepatoprotective, antimicrobial, antiviral, anticarcinogenic, antithrombotic, cardioprotective and vasodilatory activities. These actions are attributed to their antioxidant effect (Hoffmann, 2003).

Herbal medicine is increasingly emerging as an alternative for treating various diseases. The situation becomes more concerning after six decades of antibiotic use, as human and animal pathogenic bacteria have reached alarming levels of resistance to several antibiotics (Muylaert & Mainil, 2013). Additionally, it is imperative to employ the appropriate bioassays to assess the biological activities of plants (Tyihák *et al.*, 2008).

Our study focused on *Haplophyllum tuberculatum*, a member of the Rutaceae family. It is found extensively across northern Africa, particularly in the northern Sahara and southern Europe. Its distribution encompasses five distinct regions: Irano-Turanian, Mediterranean, Saharo-Arabic, and Sudano-Zambezian regions (Benchelah *et al.*, 2000). This plant is characterized by large glands containing a potent and unpleasant-smelling essence (Salvo *et al.,* 2011). Traditionally, the plant has been used to treat various conditions such as diabetes, liver diseases, ear infections, and for rheumatic pains (decoction). It is also reputed for alleviating menstrual pain, combating heart diseases, and treating scorpion stings (Hadjadj, 2015; Abdelgaleil *et al.,* 2020).

H. tuberculatum offers versatile usage options. It can be prepared as a boiled mixture or infused in milk as a remedy for stomach aches and bloating. Furthermore, when dried, it is used as a condiment to flavor goat butter and tea. The juice extracted from *H. tuberculatum* leaves is highly sought-after and is particularly used to remove warts and treat skin infections, parasitic diseases, and arthritis (Benchelah *et al.,* 2000; Raissi *et al.,* 2016). Also, the leaves of this plant infused in vinegar exhibited activity on the central nervous system. The mixture can be administered to children for the treatment of convulsions and other nervous disorders (Al-Said *et al.,* 1990).

H. tuberculatum has proven its effectiveness in traditional medicinal practices, which makes the study of its various biological activities particularly interesting and likely to require extensive research. Among these studies, Abou-Zeid *et al.* (2014) and Al-Saeghi *et al.* (2022) have demonstrated a remarkable antibacterial effect of different extracts from the aerial parts of the plant against a wide range of Gram-negative and Gram-positive bacteria, with inhibition zones of 6-12 mm and 6-20 mm, respectively. Moreover, the study carried out by Hamdi *et al.* (2021) revealed the cytotoxicity and antiviral activities of *H. tuberculatum* essential oils against Coxsackie viruses B3 and B4.

Our research is, therefore, part of this perspective and aims to extract alkaloids and polyphenols from the aerial part of *H. tuberculatum* using the maceration method, characterize these compounds by thin layer chromatography (TLC), and evaluate their antibacterial activity.

2. MATERIAL and METHODS

2.1. Plant Material

The plant material used is the aerial part of *H. tuberculatum* plant collected from Algerian fluent, in the region of Tsabit (Adrar, Algeria), during the flowering period (February-May). The plant was dried for two weeks in the open air at room temperature and protected from light.

2.2. Microbial Strains

The microbial support used is represented by Gram-positive and Gram-negative bacteria: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27953, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 700603 (laboratory strains supplied by SAIDAL-antibiotical and the Pasteur Institute of Algeria). *Klebsiella* sp. and *Proteus* sp. (strains obtained from urine cultures of hospitalized patients, provided by Bacteriology Laboratory, Hospital Krim Belkacem, Tizi Ouzou, Algeria).

2.3. Preparation of *H. tuberculatum* Extracts

The preparation of the extracts was carried out by maceration of the aerial parts of the plant. The alkaloid extract was prepared according to the method of Bruneton (1999), the powder of the plant was dissolved in a solution of hydrochloric acid (HCl 1N) and left to stir for two hours. After filtration and degreasing, the solution was placed in a separatory funnel then basified with ammoniac and extracted with chloroform. Finally, it was washed and dried over anhydrous sodium sulfate before drying in a rotary evaporator under a vacuum at 40 °C.

The extraction of total polyphenols was performed as described by Owen *et al.* (1999) through maceration of the powder in methanol for 3 days under magnetic stirring, followed by filtration. The obtained filtrate was evaporated at 40 °C using a rotary evaporator. The extract was weighed and stored at 4 °C for future uses.

2.4. Yields of Extraction and Total Polyphenols Assay

The quantitative estimation of the alkaloids and polyphenols extracts obtained from the aerial part of *H. tuberculatum* was carried out by yield determination, which is based on the ratio between the mass of the crude extract in the dry state and that of the plant material used.

Moreover, the total phenolic content of the extract was estimated using Folin Ciocalteu's method, as described by Wong *et al.* (2006), with Gallic acid as the standard. A volume of 1 mL of total polyphenolic extract of *H. tuberculatum* and different concentrations of Gallic acid was added to 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (10 times diluted in water). After 3 min, 2 mL of sodium carbonate Na_2CO_3 at 20% (w/v) was added. The solutions were then incubated in the dark for 30 min at room temperature. The absorbance reading was taken at 760 nm against a blank containing ethanol instead of Gallic acid. The result was expressed as milligrams equivalent of Gallic acid per gram of dry matter (mg GAE/g DM).

2.5. Detection of *H. tuberculatum* Compounds

The qualitative analysis of extracts was carried out by thin layer chromatography (TLC), where 10 µL of each extract was deposited at 1.5 cm from the lower edge of the plate. After drying, the plate emerged into the glass tank previously saturated with the appropriate eluent. To select the best solvent system that allowed a good separation of compounds for the two extracts studied, several mini-TLCs were carried out using aluminum plates covered with Silicagel 60 F254 (Merck) and different solvent systems (Table 1).

After development and drying, the plates were observed under UV at 365 nm, then revealed by the appropriate reagents allowing the detection of the separated compounds. Dragendorff's reagent was used to reveal alkaloids which appear in the form of orange spots, while polyphenols were revealed by ammonia vapors which give brown stains.

Table 1. Composition of the solvent systems tested (v/v) for the separation of polyphenols and alkaloids of *H. tuberculatum* by TLC.

Alkaloids	Polyphenols
S1: Chloroform-Methanol (9:1) S2: Chloroform-Acetone (9:1) S3: Hexane-Ethyl acetate (8:2) S4: Chloroform-Ethyl acetate-Acetone (5:4:1) S5: Ethyl acetate-Methanol-Distilled water (77:13:10) S6:Chloroform-Methanol-Concentrated ammonia (90:9:1) S7:Diethylether-Methanol-Concentrated ammonia (44:5:1) S8:Toluene-Acetone-Ethanol-Concentrated ammonia (40:4:8:3)	S1: Ethyl acetate-Formic acid-Acetic acid glacial-Distilled water (100:11:11:27) S2: Ethyl acetate-Methanol-Distilled water (100:13.5:10) S3: Butanol-Acetic acid-Distilled water (4:1:5) S4: Chloroform-Methanol-Distilled water (1:1:0.5)

2.6. Evaluation of The Antibacterial Activity

The disk diffusion method on solid medium as described by Balouiri *et al.* (2016) was used. Discs of filter paper (N°4) of 9 mm diameter impregnated with 60 µL of ethanol solution containing each extract corresponding to 3 mg per disc, also a disc containing the same volume of ethanol 95° (as control negative), were dried and then placed on Mueller Hinton agar previously seeded with bacterial suspension.

The Petri dishes were kept at 4 °C for three hours to promote the diffusion of extracts (Bansemir *et al.*, 2006). Afterward, they were incubated at 37 °C for 24 h. Reading was performed by measuring the inhibition diameter. The results were expressed in mm, and bacteria that showed a clear zone of inhibition were considered sensitive.

An extract is considered active when it produces an inhibition zone around the disk, and bacterial sensitivity to different compounds is classified based on the diameter of the inhibition zones as follows: $Ø \leq 8$ mm: non-sensitive strain; $9 \leq Ø \leq 14$ mm: sensitive strain; $15 \leq Ø \leq 19$ mm: highly sensitive strain; $Ø \geq 20$ mm: extremely sensitive strain (Ponce *et al.*, 2003).

The experiments were made in duplicate and repeated three times. The effect of each extract was compared to Amikacin (30 µg), Kanamycin (30 UI), Oxacillin (5 µg), amoxicillin (25 µg), Penicillin G (6 µg), Amoxicillin + Clavulanic acid (25 µg), Chloramphenicol (30 µg), Colistin (50 µg), Vancomycin (30 µg), and Doxycyclin (30 UI).

Statistical analysis was performed using XLSTAT 7.5.2. (2007). Data were analyzed statistically using one-way analysis of variance (ANOVA) (for $p<0.05$*;* differences were considered to be statistically significant).

2.7. Determination of The Minimum Inhibitory Concentration (MIC)

The MIC was estimated by the dilution method on a solid medium, as described by Amhis *et al.* (2001), with few modifications. The technique consists of adding, in sterile test tubes, 9 mL of Mueller Hinton agar in superfusion, with different quantities of polyphenolic and alkaloid extract of *H. tuberculatum*. The final concentrations of each extract in the culture medium were: 0.625, 1.25, 2.5, 5, 10, and 20 mg/mL. Once mixed, the content of each tube was poured into a Petri dish. After solidification, each dish was inoculated simultaneously with several bacterial strains in the form of parallel strips. A control dish containing 1 mL of ethanol 95° and 9 mL of Mueller Hinton agar was inoculated in the same way. The MIC is given by the first concentration, which removes any apparent culture on the agar.

3. RESULTS

3.1. Yield of Extracts and Total Polyphenols Content

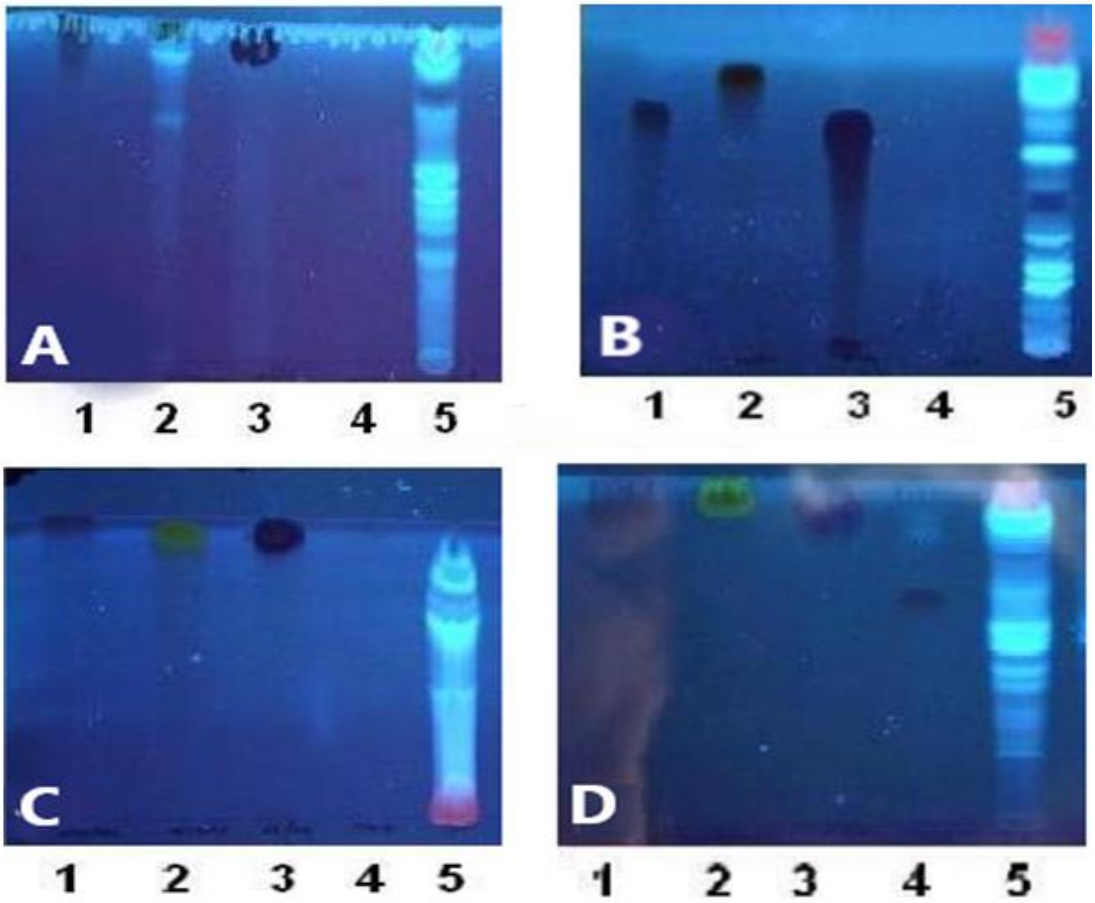
The extraction of polyphenols and alkaloids of *H. tuberculatum* by maceration method showed that the plant is rich in phenolic and alkaloid compounds. The yield was 8.39 ± 0.04% for total polyphenols against only 0.37 ± 0.01% for alkaloids. Using the Folin Ciocalteu's reagent, the content of polyphenols was estimated at 74.45 ± 1.43 mg GAE/g DM.

3.2. Qualitative Analysis of *H. tuberculatum* Extracts

3.2.1. *Characterization of the total polyphenolic extract*

Figure 1 shows the chromatograms obtained with the various solvent systems as the mobile phase under a UV lamp at 365 nm. The results reveal that the solvent system S3 (n-butanol, acetic acid, distilled water (4:1:5) provided poor separation for the compounds of *H. tuberculatum* extract, as well as for the standard molecules (Figure 1, C). The solvent system S1 (ethyl acetate, formic acid, glacial acetic acid, distilled water (100:11:11:27)) enabled a good separation of the total polyphenolic extract and well-defined spots for the standard molecules (Figure 1, A). In contrast, solvent system S2 (ethyl acetate, methanol, distilled water (100:13.5:10)) yielded a satisfactory separation of the crude polyphenolic extract, showing distinct spots and efficient migration of standard molecules (Figure 1, B). Similar observations were made with solvent system S4 (chloroform, methanol, distilled water (1:1:0.5)), but with a lower number of spots in the crude polyphenolic extract (Figure 1, D).

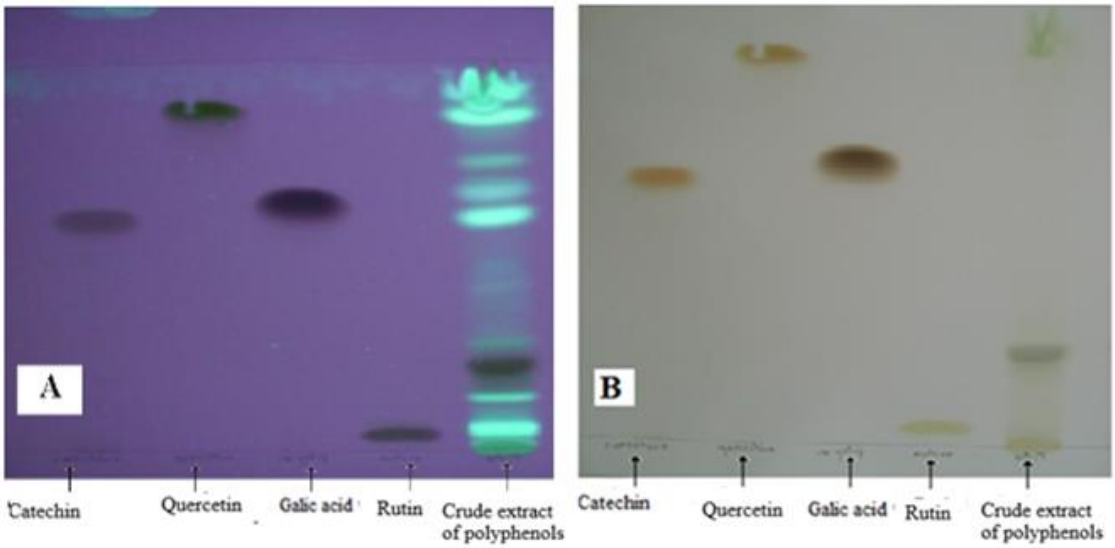
Figure 1. Revelation under UV at 365 nm of the chromatograms of polyphenolic extract from *H. tuberculatum* and standard molecules obtained with the four solvent systems tested. **1**: Catechin, **2**: Quercitin, **3**: Gallic Acid, **4**: Rutin, **5**: Total polyphenols extract. **A, B, C** and **D**: Chromatograms developed with the solvent system S1, S2, S3 and S4, respectively.



Based on these findings, we selected the combination of ethyl acetate, methanol, and distilled water (100:13.5:10) as it provided the best separation of the total polyphenolic extract of *H. tuberculatum* and the standard molecules used.

Subsequently, a new separation was performed on an entire 20 cm × 20 cm plate to ensure a good identification of the different spots and their colors (Figure 2). The results revealed that the total polyphenols extracted by maceration in methanol exhibited blue or brown fluorescence bands. Exposure of the chromatogram to ammonia vapors unveiled the presence of darker brown-colored spots. According to the obtained observations and with comparison to standard molecules, traces of quercetin, catechin, and rutin were found in our plant's polyphenolic extract.

Figure 2. Chromatograms of the polyphenolic extract of *H. tuberculatum* and the standard molecules separated with the solvent system S2: ethyl acetate-methanol-distilled water (100:13.5:10). **A**: Observation under UV at 365 nm. **B**: Revelation with ammonia vapors.



3.2.2. *Characterization of the alkaloid extract*

The results of observation under UV at 365 nm (Figure 3) of *H. tuberculatum* alkaloids carried out with the eight solvent systems revealed several fluorescent spots, the number of which varied according to the composition of the mobile phase. Solvent systems S1, S2, S4, S6, and S8 (composition: see Table 1) were able to cause the alkaloid extract of *H. tuberculatum* with varying degrees. The best separation was observed with the S8 system, which gave the greatest number of fluorescent spots. However, the chromatograms of the solvent systems S3, S5, and S7 (composition: see Table 1) showed streaks.

After revealing the chromatograms with Dragendorf's reagent, the results showed characteristic orange spots of alkaloids in all chromatograms (Figure 4). Nevertheless, it was the S8 system (toluene-acetate-ethanol-concentrated ammonia 40:4:8:3) that gave the maximum number of these spots (about ten spots), thus suggesting that *H. tuberculatum* is quite rich in alkaloid compounds of different natures.

Figure 3. Chromatograms of the alkaloid extract of *H. tuberculatum* observed under UV at 365 nm and obtained with the different solvent systems.

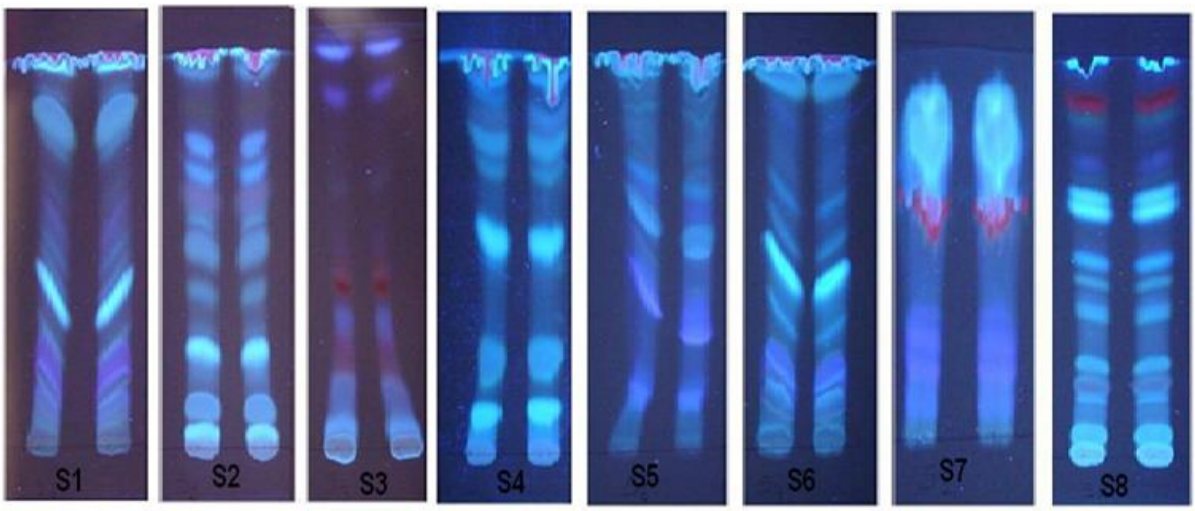
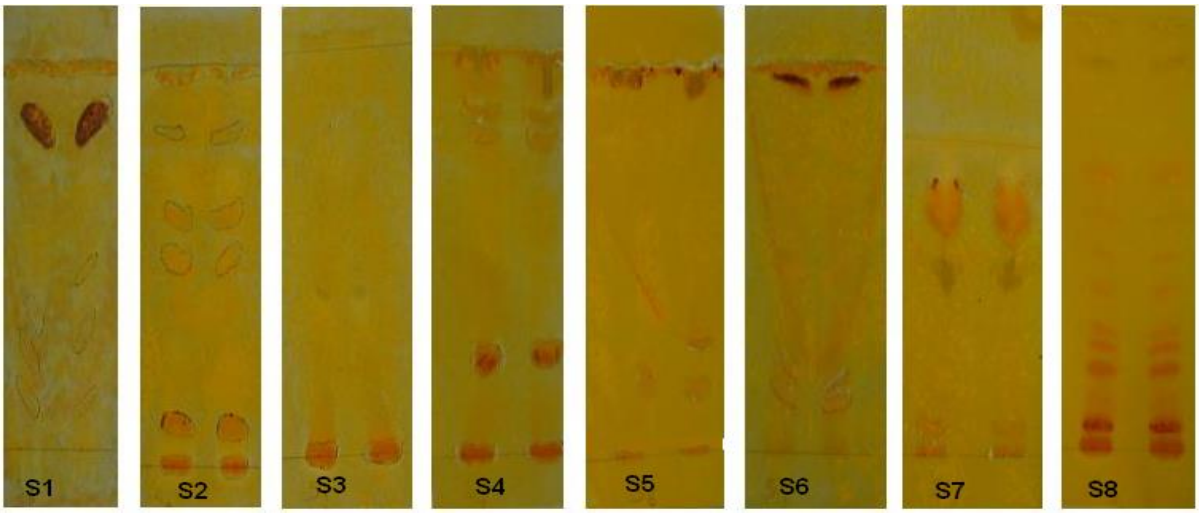


Figure 4. Chromatograms of the alkaloid extract of *H. tuberculatum* revealed by Dragendorf 's reagent and obtained with the different solvent systems.



3.3. Antibacterial Activity of *H. tuberculatum* Extracts

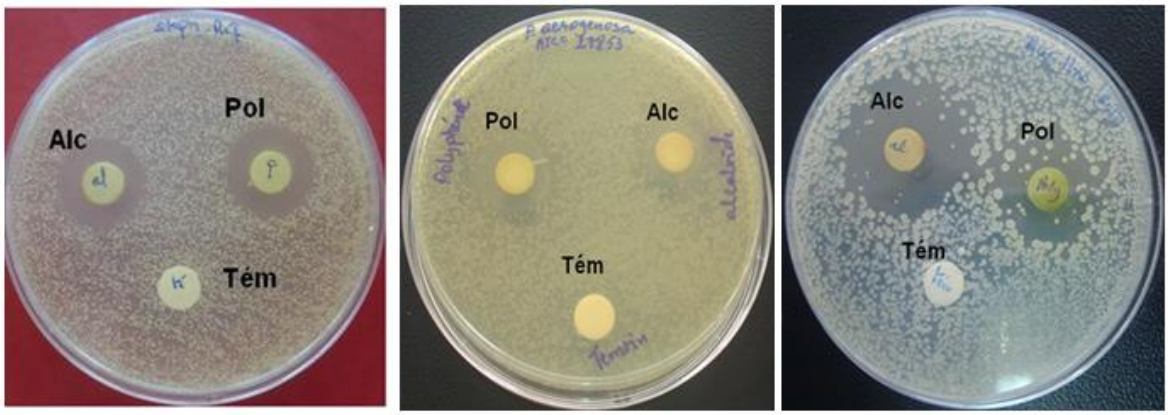
The results (Table 2 and Figure 5) show that some bacterial strains are sensitive to both extracts, the Gram-positive strains represented by *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 with halos of inhibition around 19 ± 1.00 mm and 25.66 ± 4.04 mm, respectively, for alkaloids, and 20.66 ± 1.52 mm and 22 ± 2.00 mm for polyphenols. The sensitive Gram-negative strains were represented by *P. aeruginosa* ATCC 27953 with zones of inhibition of 14 ± 1.00 mm for alkaloids and 17 ± 2.00 mm for polyphenols. Whereas, the other Gram-negative strains tested: *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *Proteus* sp. and *Klebseilla* sp. were all resistant.

Table 2. Diameter of inhibition zones induced by *H. tuberculatum* extracts (3 mg of extract/disc) and Amikacin (30µg) as positive control using disk diffusion method.

	Diameter of inhibition zones (mm)		
Strain	Polyphenols	Alkaloids	Amikacin (30µg)
S. aureus ATCC 25923	20.66 ± 1.52	19.00 ± 1.00	24.33 ± 2.08
P. aeruginosa ATCC 27953	17.00 ± 2.00	14.00 ± 1.00	22.16 ± 1.25
B. subtilis ATCC 6633	22.00 ± 2.00	25.66 ± 4.04	34.00 ± 2.00
E. coli ATCC 25922	/	/	NT
K. pneumoniae ATCC 700603	/	/	NT
Proteus sp.	/	/	NT
Klebsiella sp.	/	/	NT

/: Absence of inhibition, ND: not tested.

Figure 5. The appearance of cultures and inhibition zones in the presence of polyphenolic and alkaloid extracts from *H. tuberculatum*, obtained by the disk diffusion method on Mueller Hinton agar (**Tém**: negative control, **Pol**: total polyphenols, **Alc**: alkaloids).



S. aureus ATCC25923 *P. aeruginosa* ATCC27953 *B. subtilis* ATCC6633

In addition, several antibiotic tests were carried out to evaluate the sensitivity of the three strains. The results of the antibiogram demonstrated that Amikacin (30 µg) exhibited the highest effect against all sensitive strains to alkaloid and polyphenol extracts from the plant *H. tuberculatum.* Therefore, it was chosen as the positive control, where its antibiotic effect was proved to be superior to that of the tested plant extracts. The inhibition zones of Amikacin are mentioned in Table 2. Analysis of variance with a confidence level of 95% ($p<0.05$) revealed that the difference between the effect of polyphenolic and alkaloid extracts of *H. tuberculatum* and the control (Amikacin) is significant for the three sensitive strains.

3.4. The Minimum Inhibitory Concentration of Alkaloid and Polyphenolic Extracts

The results of the MIC (Table 3) show that the alkaloids of *H. tuberculatum* were more active than its polyphenols, with MICs between 0.625 to 10 mg/mL for alkaloids (Figure 6) and from 5 to 20 mg/mL for polyphenols (Figure 7). Moreover, *B. subtilis* ATCC 6633 was the most sensitive strain to both extracts, followed by *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27953.

Table 3. MICs of polyphenols and alkaloids extracts from *H. tuberculatum* against some bacterial strains.

	MIC (mg/mL)	
Bacterial strains	Polyphenols	Alkaloids
S. aureus ATCC 25923	10.0	0.625
P. aeruginosa ATCC 27953	20.0	10.00
B. subtilis ATTC 6633	5.00	0.625

Figure 6. The appearance of bacterial cultures on Mueller Hinton agar in the presence of increasing doses of *H. tuberculatum* alkaloids. (**B**: *B. subtilis* ATCC 6633, **St**: *S. aureus* ATCC 25923, **Ps***: P. aeruginosa* ATCC 27953). **1**: MH agar + alkaloids 0 mg/mL, **2**: MH agar + alkaloids to 0.625 mg/mL, **3**: MH agar + alkaloids to 1.25 mg/mL, **4**: MH agar + alkaloids to 2.5 mg/mL, **5**: MH agar + alkaloids to 5 mg/mL, **6**: MH agar + alkaloids to 10 mg/mL.

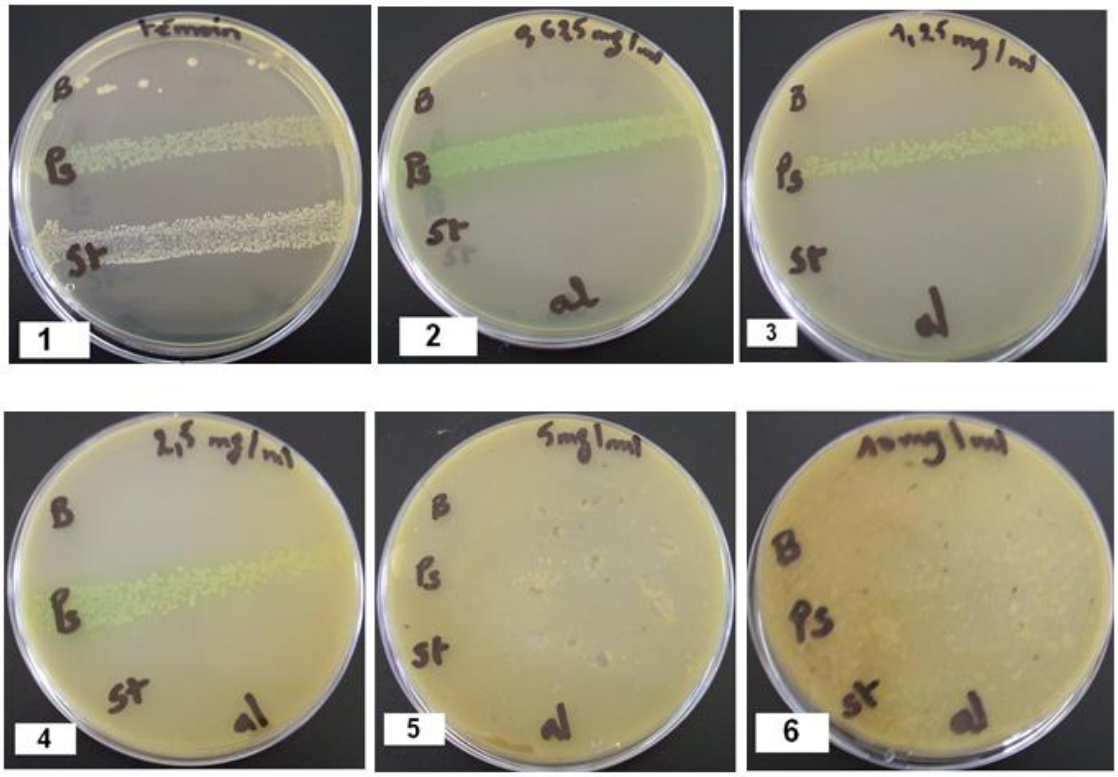
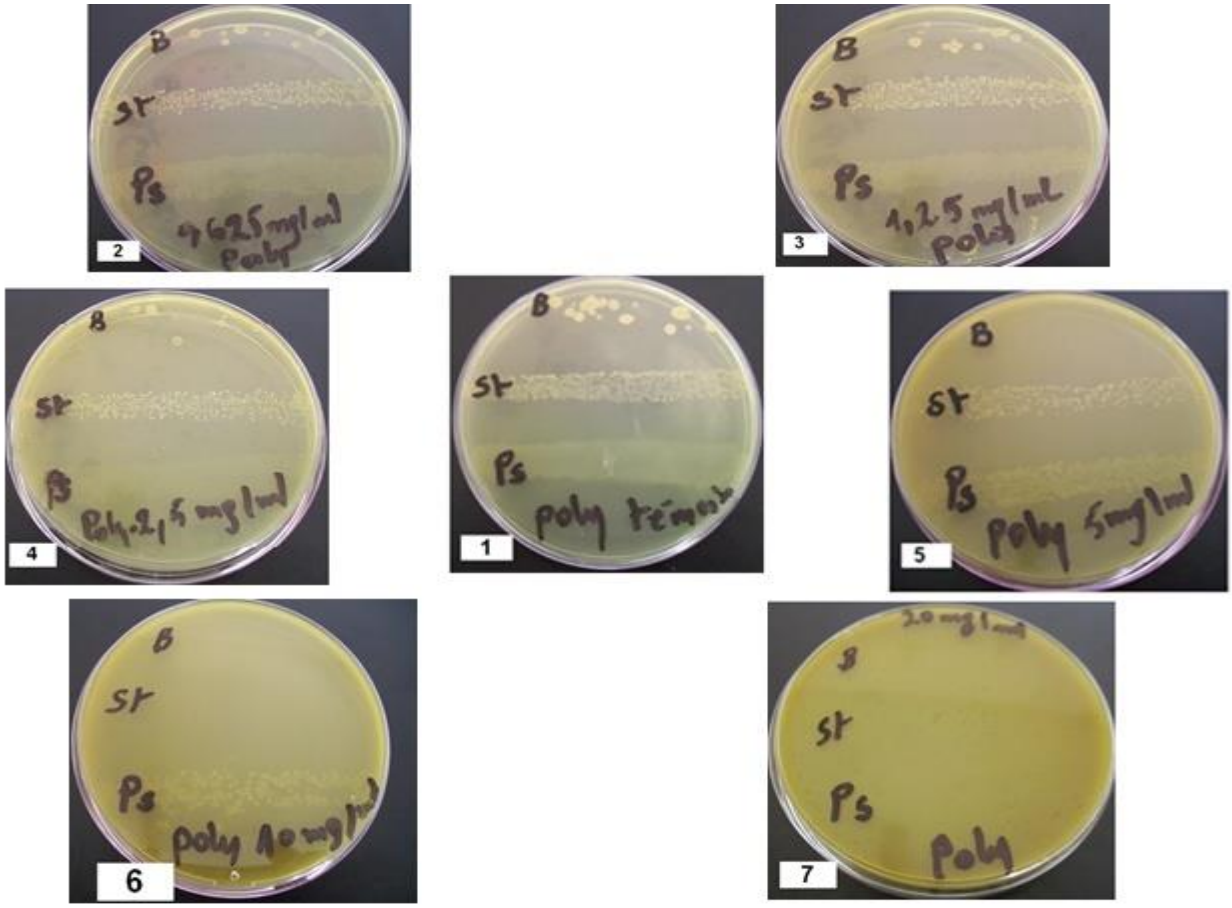


Figure 7. The appearance of bacterial cultures on agar Muller Hinton (MH) in the presence of increasing doses of *H. tuberculatum* polyphenols. (**B**: *B. subtilis* ATCC 6633, St: *S. aureus* ATCC 25923, Ps: *P. aeruginosa* ATCC 27953). **1**: MH agar + polyphenols at 0 mg/mL, **2**: MH agar + polyphenols to 0.625 mg/mL, **3:** MH agar + polyphenols to 1.25 mg/mL, **4**: MH agar + polyphenols to 2.5 mg/mL, **5**: MH agar + polyphenols to 5 mg/mL, **6**: MH agar + polyphenols to 10 mg/mL, **7**: MH agar + polyphenols to 20 mg/mL.



4. DISCUSSION and CONCLUSION

According to Al-Brashdi *et al.* (2016), the aerial part of *H. tuberculatum* is rich in phenolic compounds that could play a vital role in discovering natural antioxidants. The same authors have observed that the most polar fractions (methanol) have the highest content of phenols (561.22 mg GAE/g), which is higher than the one found during our work (74.45 ± 1.43 mg GAE/g DM). These variations in results were related to several factors: climatic and environmental factors (region, soil, salinity, etc.), genetic factors, storage duration, period and stage of the plant development (Miliauskas *et al.*, 2004; Bouzid *et al.*, 2011; Touati *et al.*, 2018). In addition, Lee *et al.* (2003) indicated that the extraction and quantification methods can influence the estimation of the total phenols content.

The characterization of polyphenolics and alkaloids of *H. tuberculatum* by thin-layer chromatography and their observation under UV at 365 nm revealed blue and brown fluorescence bands. According to Dohou *et al.* (2003) and Diallo (2005), the bands of blue fluorescence would correspond to phenol acids or the presence of coumarins, while those colored brown would correspond to flavonols and flavones.

The work of Abdelkhalek *et al.* (2020) proved the presence of gallic acid, catechin, quercetin and rutin in the crude ethanolic extract of *H. tuberclatum* following High Performance Liquid Chromatography (HPLC) analysis, which confirms our results.

The revelation of chromatograms using ammonia vapors for polyphenols and Dragendorf's reagent for alkaloids showed several characteristic spots of the two secondary metabolites. Indeed, the work of Hamdi *et al.* (2018) showed the presence of phenols, tannins, alkaloids, flavonoids, terpenoids, sterols, cardiac-glycosides, saponosides, lipids and fixed oils in the crude methanolic extract.

Furthermore, the work performed by Al-Shamma (1979), Sheriha (1984) and Al-Yahya *et al.* (1992) on the same plant showed that the alkaloids of this plant are very variable; and represented by haplotubinone, haplotubin, tuberine, tubacetin, tubasenicin, quinolines, flindersin, skimianine and evoxin.

Concerning the antibacterial activity of the two extracts of the plant, it was found that all the strains sensitive to the alkaloid extract are simultaneously sensitive to the polyphenolic extract. However, the degree of sensitivity is variable, where the alkaloids are more effective. This can be explained by the presence of common substances between the two extracts. Moreover, the presence of phenolic acids was confirmed in the alkaloid extract by the appearance of blue bands after its observation under UV. Likewise, the polyphenolic extract may contain alkaloids in the form of soluble salts in methanol.

Regarding the sensitivity of Gram-positive bacteria compared to Gram-negative towards plant extracts, several studies have demonstrated this effect (Hayouni *et al.*, 2007; Shan *et al.*, 2007; Falleh *et al.*, 2008; Çolak *et al.*, 2009). This and according to the same authors, is due to differences in the outer layer composition of bacteria. The Gram-negative strains have more, outer membrane, which is composed of phospholipids, proteins and lipo-polysaccharides, impermeable to most molecules.

The comparison of our results with those of previous studies showed that the antibacterial effect of polyphenolic and alkaloid extracts of *H. tuberculatum* is partially consistent with the work of Sheriha (1984) and Al-Rehaily (2001) performed on the same plant. These authors reported that Tuberin isolated from this plant collected in Iraq exerts an antibacterial effect against *S. aureus* and *E. coli* with doses of 0.10 to 1.0 mg/mL. We also noted that the phenolic and alkaloid substances of this plant have less effect on Gram-negative bacteria than its essential oils, where the work carried out by Al-Burtamani *et al.* (2005), showed that the essential oils of *H. tuberculatum* inhibit the growth of *E. coli* and *Salmonella choleraesuis.*

The phytochemical study established by Abdelgaleil *et al*. (2020) led to the isolation of three alkaloids (skimmianine, vulcanine, and evoxine) from the aerial parts of *H. tuberculatum*. Skimmianine was among the most potent inhibitors against the phytopathogenic bacteria: *Rhizobium radiobacter*, *Ralstonia solanacerum* and *Pectobacterium carotovorum*, with MIC value of 62.5 µg/mL.

Compared to other species of the *Rutaceae* family, the work of Singh *et al.* (2020) shows the antibacterial efficacy of methanolic extract from *Zanthoxylum armatum* leaves against Gram-positive and Gram-negative bacterial strains with an inhibition zone of 17.67 mm for *S. aureus* and 12.33 mm for *E. coli*. According to work published by Pavić *et al.* (2019), the best antibacterial activity of *Ruta graveolens* extracts was observed against *P. aeruginosa* with the lowest MIC (62.5 µg/mL). While the highest MIC (125 µg/mL) was observed against *E. coli, B. subtilis* and *S. aureus.*

Indeed, alkaloids are known for their high antibacterial effect. The mechanism action of antibacterial alkaloids intervenes through blocking cell division, by preventing the synthesis of microtubules and mitotic spindle formation, which is essential for cell division (Karou, 2005).

The antimicrobial activity of polyphenols may involve complex mechanisms such as inhibition of cell walls, cell membranes, protein synthesis and nucleic acid metabolism (Aboshora *et al.*, 2014). According to Daglia (2012), flavonols have antibacterial activity

against several Gram-positive bacteria. At equivalent concentrations, some flavonoids have greater antibacterial activity than tetracycline or vancomycin. Similarly, the mechanism of polyphenol toxicity against microorganisms may be related to inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other interactions to inactivate adhesion proteins, and cell envelope transport proteins, no specific interactions with carbohydrates, among others (Alvarez *et al.*, 2010).

In conclusion, the present research confirms that *H. tuberculatum* is more enriched in polyphenols than in alkaloids. Qualitatively, thin-layer chromatography showed that this plant contains a wide range of alkaloids and polyphenols, revealed by several spots on the chromatograms. The antibacterial activity was interesting and effective with both extracts, especially on Gram-positive strains. Nevertheless, on Gram-negative strains, the effect was observed only with *P. aeruginosa* ATCC 27953. However, the other Gram-negative strains such as *Escherichia coli* ATTC 25922, *Klebsiella pneumoniae* ATTC 700603, *Proteus* sp., and *Klebsiella* sp., were all resistant to both extracts. Our study showed that *H. tuberculatum* extracts can be used as promising new sources of antibacterial molecules in the treatment of bacterial infections. Therefore, purification and identification of both extracts by instrumental techniques would be necessary.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

All authors contributed to the study conception and design. The plant was provided by **Fatma Acheuk**. Material preparation, experiments and data analysis were performed by **Djamila Djouahra-Fahem**. The first draft of the manuscript was written by **Djamila Djouahra-Fahem, Souhila Bensmail** and **Razika Bouteldja**. **Fethia Fazouane** and **Fatma Acheuk** supervised the work. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Research Article

Chemical composition, antioxidant, and antimicrobial activities of Bangladesh-origin Jhum-cultivar basil (*Ocimum basilicum* L.) essential oil

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Abstract: Essential oil (EO) from the sweet basil (*Ocimum basilicum* L.) grown in the Jhum cultivations located in Bangladesh was screened for chemical composition, antioxidant, and antimicrobial activities. EO yield from the Jhum-cultivar *O. basilicum* was 1.55% (v/w). Analysis of EO indicated the presence of several bioactive compounds, among which *Geranial* (35.5%) and *cis-citral* (26.2%) are of significant content. The EO showed antioxidant activities inhibiting DPPH radical with a mean value of 45.7% at 2.4 mg mL^{-1} of EO. The EO has susceptibility against Gram-positive and Gram-negative bacteria (*Escherichia coli*, *Salmonella* Typhi, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus cereus*, and *Micrococcus* spp.), with a notable activity against the Gram-positive bacteria.

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1. INTRODUCTION

Ocimum species (basil), a native herb to Asia and also grows wild in tropical and sub-tropical regions worldwide, is often referred to as the *king of herbs* owing to its popularity (Makri & Kintzios, 2008). Sweet basil (*Ocimum basilicum* L.) is the most commonly grown species of the *Ocimum* genus and also represents a significant fraction of commercial basil cultivars available in the market (Calin-Sanchez *et al*., 2012; Jirovetz *et al*., 2003; Makri & Kintzios, 2008; Shahrajabian *et al*., 2020). As a culinary herb, basil induces distinctive flavor in Iranian, Italian, Chinese, and Indian cuisines (Akbari *et al*., 2018; Makri & Kintzios, 2008; Shahrajabian *et al*., 2020). The medicinal uses and potential health benefits of *O. basilicum* in traditional and

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modern medicine have been reported (Kaefer & Milner, 2008; Twilley *et al*., 2018; Zhan *et al*., 2020). *O. basilicum* is used in ethnomedical practices to treat coughs, stomach-related diseases, involuntary muscle spasms, parasitic worms, and skin infections (Labra *et al*., 2004; Shirazi *et al*., 2014). The essential oil (EO) from *O. basilicum* contains several bioactive chemicals, such as flavonoids, phenol derivatives, phenyl-propanoids, triterpenoids, steroids, and steroidal glycosides (Rezzoug *et al*., 2019; Siddiqui *et al*., 2012; Singh *et al*., 2018; Złotek *et al*., 2017). The constituents in *O. basilicum* EO are known to possess antioxidative, anti-inflammatory, antispasmodic, antibacterial, antifungal, and antioxidant properties (Ahmad *et al*., 2016; Ahmed *et al*., 2019; Burt, 2004; Miguel, 2010; Stanojevic *et al*., 2017; Tankeo *et al*., 2014; Złotek *et al*., 2016). However, *O. basilicum* EO's composition varies depending upon the origin of the plant (Bilal *et al*., 2012; Da-Silva *et al*., 2003), plant genotype, soil and climatic conditions, growing technique, harvest time, irrigation as well as fertilization (Mith *et al*., 2016; Muzika *et al*., 1989).

O. basilicum L. is a popular herb among the indigenous communities living in the Chittagong Hill Tracts (CHT) of Bangladesh. It is consumed both as a food ingredient and in ethnomedicine. The herb is produced via the traditional shifting cultivation farming technique (slash-and-burn agriculture), commonly known as *Jhum* cultivation. The chemical constituents and pharmacological benefits of various *O. basilicum* cultivars have been reviewed (Bakkali *et al*., 2008; Miguel, 2010; Shahrajabian *et al*., 2020; Swamy *et al*., 2016; Zhan *et al*., 2020). However, to our knowledge, EO from any Jhum-cultivar *O. basilicum* variety has not been screened before. Hence, the current study aims to assess the chemical composition, antimicrobial, and antioxidant properties of the EO derived from *O. basilicum* species collected from the Jhum cultivations in CHT, Bangladesh.

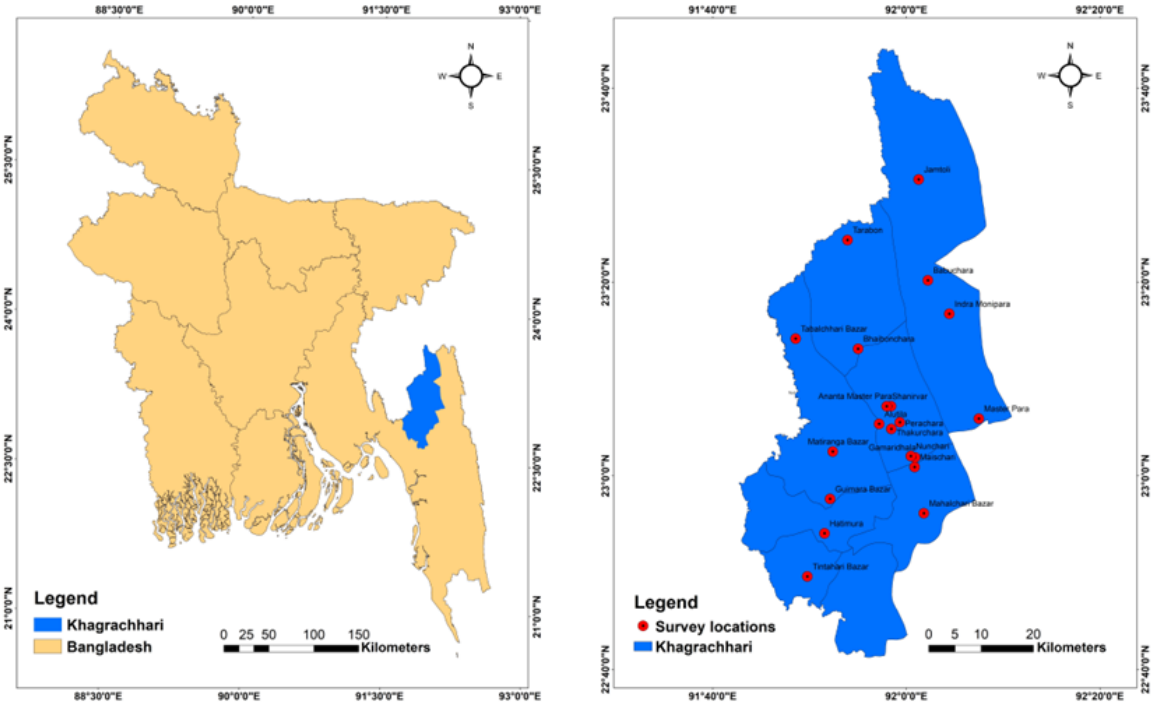
2. EXPERIMENTAL

2.1. Materials

2.1.1. *Sample*

The *O. basilicum* species were collected from the *Khagrachhari* hill district in Bangladesh. The study location and sampling points are shown in Figure 1.

Figure 1. Location map showing the sampling points.



2.1.2. *Chemicals and reagents*

2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Shanghai Shenmin Industrial Co., Shanghai, China), tetrazolium chloride (Sigma-Aldrich, St. Louis, MO), and dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) were used without further purification.

2.1.3. *Microorganisms*

The food-borne pathogens *Staphylococcus aureus* (ATCC6538), *Bacillus cereus* (ATCC14574), *Micrococcus* spp. (ATCC4698), *Salmonella* Typhi (ATCC14028), *Vibrio cholerae* (ATCC14035), and *Escherichia coli* (ATCC25922) were used for antimicrobial activity monitoring.

2.2. Methods

2.2.1. *Separation of EO*

Fresh leaves of *O. basilicum* were subjected to hydrodistillation for 2 h, assisted by the Clevenger apparatus. Next, the distilled EO was dried on anhydrous sodium sulfate. Afterward, the filtrate was transferred into a sealed vial and preserved at 4 °C for further analyses.

2.2.2. *Antimicrobial activity analysis*

The disc diffusion method (Kim *et al*., 1995) was used to study the activity of EO against the above-mentioned food-borne bacteria. The fresh culture of organisms was adjusted at 1×10^8 CFU mL^{-1} by comparing with the 0.5 Mc Farland standard (optical density, 0.80–0.12). The cultured organism was swabbed into the Mueller-Hinton Agar (MHA) plates. The sterile paper disc (dia, 6 m) was soaked with different EO concentrations (10, 15, 20 µL of 1:1 (v/v) dilution with 0.5% DMSO). The air-dried cultures were incubated at 37 °C for 24 h in MHA plates. One filter paper disc was placed per Petri dish to avoid a possible additive activity. DMSO (0.5%, 10 µL) was used as the negative control, and standard antibiotic chloramphenicol (10 µg) was used as the positive control. Obstruction zones were measured using a transparent ruler on an mm scale. If the zone of inhibition is measured >15 mm, it will be termed as *strongly inhibitory*, 10 to 15 mm as *moderately inhibitory*, and <10 mm as *not inhibitory*.

2.2.3. *Bactericidal concentration analysis*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were tested according to the broth dilution method (Mith *et al*., 2016; Sakkas *et al*., 2018; Veiga *et al*., 2019). The stock solution of EO was prepared by diluting EO with 2 to 5% DMSO in a ratio of 1:1. 10^{-1} dilution of Mueller-Hinton Broth (MHB) was prepared by mixing 4.5 mL of sterilized MHB with 0.5 mL of freshly prepared stock solution. The micro-dilution tests were performed in sterile round-bottomed 96-well microplates. First, 100 µL of 10^{-1} MHB was transferred into 1st wells of the microtiter plate. Next, 50 µL of previously prepared sterile MHB was moved into the subsequent 2–12 wells of a microtiter plate. Two-fold dilution was performed by transferring 50 µL from the 1st to the 11th well. (12th well: drug-free well/ growth control). Next, 10^{-2} dilutions of prepared 0.5 Mc Farland standard inoculation (1.5×10^8 CFU mL^{-1}) of six human pathogenic bacteria were prepared. From this dilution, 50 µL of inoculum was transferred from the 1st to 12th wells (except the 11th one used for a negative control). Then, the microtiter plate was incubated at 37 °C for 18 to 24 h. MIC was recorded according to the inhibition of visible growth. To determine the MBC, we picked the area where no increase was observed.

2.2.4. *Antioxidant activity analysis*

The antioxidant activity of *O. basilicum* EO was examined by the DPPH radical scavenging method (Mensor *et al*., 2001). The antioxidant activities of EO were assessed by measuring the ability to scavenge DPPH stable radicals (Saha *et al*., 2017). EO sample (1.5 mL) was prepared

in different concentrations using methanol. A reaction mixture of 0.5 mL consisting of a 0.5 M acetic acid buffer solution of pH 5.5 and 1 mL of 0.2 mM DPPH in methanol was prepared. The sample solution and reaction mixture were combined and kept in the dark for 30 min after incubation at room temperature. A chemical reaction occurred between DPPH and EO samples. The unused DPPH concentration was analyzed at 517 nm using a UV-1601 spectrometer (Shimadzu, Kyoto, Japan).

The reference standard was ascorbic acid. DPPH radical scavenging concentration was computed using the following equation.

$$\text{DPPH radical scavenging concentration (\%)} = [1 - \frac{A_{\text{sam}}}{A_{\text{cont}}} \times 100]$$

Here, A_{cont} = absorbance of the control, A_{sam} = absorbance of sample solution. Then, % inhibition was plotted against the used respective concentrations.

2.2.5. *Chemical composition analysis*

The EO's gas chromatographic-mass spectrometric (GC-MS) analysis was performed using GC system 7899A with MS detector 5975C (Agilent Technologies, Santa Clara, CA). A fused silica capillary column of (5% phenyl)-methylpolysilloxane (HP-5MS) (length, 30 m; diameter, 0.250 mm; film, 0.25 μm) was used. The GC parameter was set as follows: inlet temperature, 250 °C; oven temperature, 60 °C for 0 min, 10 °C per min to 200 °C for 2 min, and 12 °C per min to 300 °C for 5 min. The total analysis time was 29.33 min with a column flow rate of 2.43 mL min^{-1} helium gas.

The mass spectrometer (MS) was an electron ionization type. MS quad temperature was set at 150 °C, and the source temperature was set at 230 °C. The detector voltage was 0.2 kV, and the mass range was 50–550 m/z. The Total Ionic Chromatogram (TIC) was used to determine each compound's peak area and percentage concentration.

3. RESULTS AND DISCUSSION

3.1. Composition of EO

It has been reported that basil EO yields can vary significantly from 0.07 to 1.92% in different basil assays (Zheljazkov *et al*., 2008). The yield of light yellow EO obtained with a characteristic pleasant aroma was 1.55% v/w. Considered EO content was expressed as mL per 100 g raw sample.

The Jhum-cultivar *O. basilicum* EO has 19 bioactive compounds representing 99.95% of the total mass (Figure 2 and Table 1), with a significant presence of geranial (35.5%), followed by cis-citral (26%), cyclopentanol (5%), and linalool (4.94%). Hence, the biophysical and biological properties of EOs depend on the identified 19 compounds.

A previous study on the Bangladesh-origin *O. basilicum* EO (sample collection location: Dhaka) showed methyl chavicol and gitoxegenin as the major constituents (Hossain *et al*., 2010), while geranial has been reported as the significant EO constituent in several *O. basilicum* varieties (Shahrajabian *et al*., 2020; Zhan *et al*., 2020). Nerol and geraniol are oxidative forms of neral and geranial (Patora *et al*., 2003). Geraniol induces antibacterial and antifungal properties (Lira *et al*., 2020). It has been assumed that geranial mixed with other constituents to form geraniol and thus accelerated the antibacterial properties of Jhum-cultivar *O. basilicum* EO. Turpentine alcohols, such as linalool, exhibit potent antibacterial activity against bacterial strains (Uysal *et al*., 2011). In India, under different climatic conditions, it has been reported that *O. basilicum* EO is also rich in linalool, geraniol, geranial, caryophyllene, and limenone (Maurya *et al*., 2022).

Figure 2. GC-MS spectrum of Bangladesh-origin Jhum-cultivar *O. basilicum* EO.

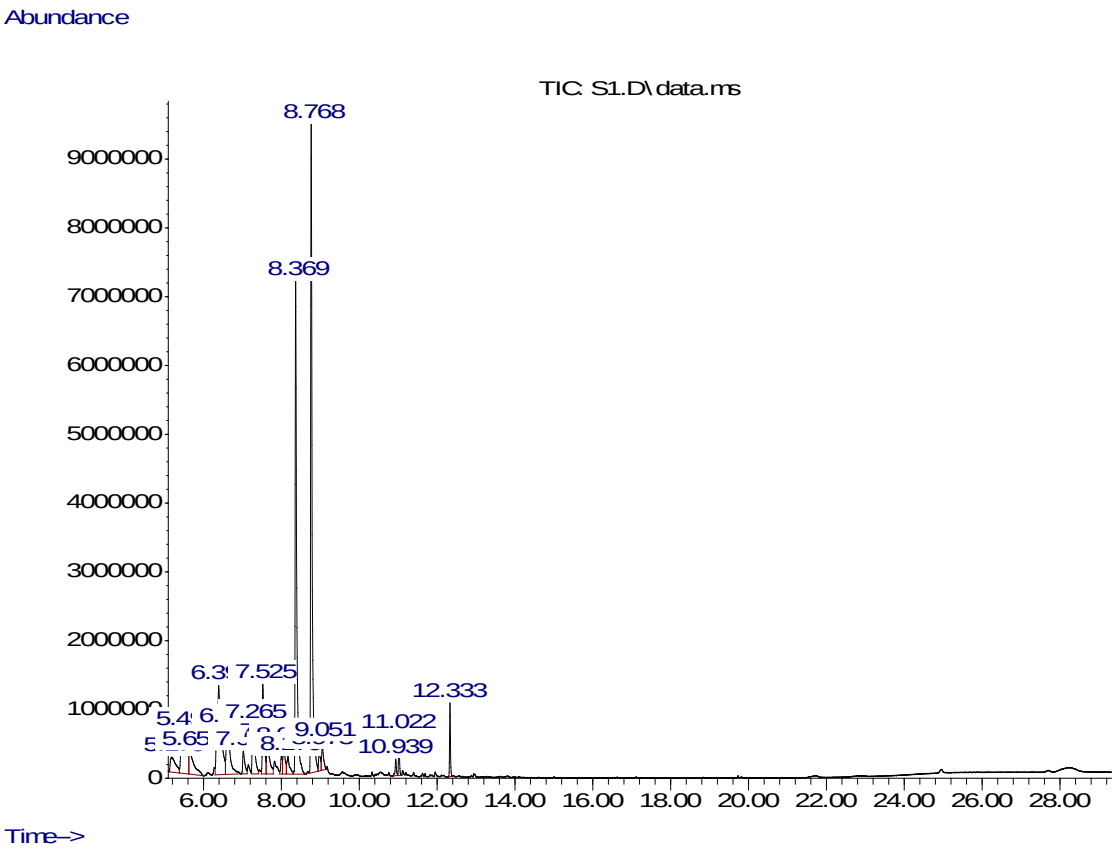


Table 1. Major constituents in Bangladesh-origin Jhum-cultivar *O. basilicum* EO.

Compound name	RT*	Absolute height	%Concentration
Mentha-1,4,8-triene	5.178	304652	1.12
Thymene	5.493	671859	2.46
l-Phellandrene	5.648	388687	1.42
Linalool	6.392	1349647	4.94
Santolina triene	6.603	716933	2.62
Geraniol acetate	7.027	390986	1.43
Limonene oxide	7.267	785715	2.88
Cyclopentanol	7.524	1369252	5.01
Fumaric acid	7.645	507564	1.85
Ethanone	8.005	321851	1.18
Chrysanthenone	8.062	448254	1.64
Camphene	8.171	315577	1.16
cis-Citral	8.371	7152777	26.19
Geranial	8.766	9693337	35.50
Piperidine	8.972	406767	1.48
Pentanamide	9.052	520585	1.90
Caryophyllene	10.941	272579	0.99
Bergamotene	11.021	621629	2.28
Bisabolene	12.331	1065430	3.90
			Total, 99.95%

*RT, Retention time

The low contents of bergemotene, bisabolene, caryophyllene, llimonene oxide, and santolena triene have contributed to the antibacterial activity. However, it is also possible that the rest secondary elements may be involved in some form of synthesis with other active compounds (Marino *et al*., 2001). The composition of Jhum-cultivar *O. basilicum* EO shows significant differences from the same species cultivated in Pakistan, Kenya, Iran, and Serbia, Egypt (Table 3). Such differences in composition can be attributed to the geographical diversity and variations in the drying process or EO extraction approach.

3.2. Antimicrobial Activity

The effect of *O. basilicum* EO on Gram-positive and Gram-negative bacteria growth at three different volumes (1:1 v/v dilution with DMSO) was studied. The antibacterial behavior of Jhum-cultivar *O. basilicum* EO is shown in Table 2. *O. basilicum* EO has shown a more substantial impact on Gram-positive bacteria, except for *V. cholerae*, than on Gram-negative bacteria, as evident from the MIC and MBC values (Tables 3 and 4). *Micrococcus* spp. showed the highest zone of inhibition (Figure S1. See Appendix A. Supplementary information). The MIC and MBC values of *B. cereus* (0.69 and 1.39 mg mL^{-1}) were minimum, while *S.* Typhi and *E. coli* showed maximum MIC and MBC (5.59 and 11.18 mg mL^{-1}). The cell wall of Gram-negative bacteria can prevent the penetration of antimicrobial compounds of EO (Burt, 2004; Nazzaro *et al*., 2013), which might be the reason for higher resistance to EO by Gram-negative bacteria than Gram-positive bacteria. The results of this study are similar to other investigations that have already been published (Anand *et al*., 2011; Hossain *et al*., 2010; Hussain *et al*., 2008; Sakkas *et al*., 2018; Silva *et al*., 2015; Stanojevic *et al*., 2017).

The impact of origin diversity on *O. basilicum* EO's antimicrobial behavior has also been observed (Table 4). For instance, *O. basilicum* EO collected from India exhibits MIC 12.5 and 6.25 mg mL^{-1} for *S. aureus* and *E. coli*, respectively, which were higher than the findings from the current work (Anand *et al*., 2011; Sakkas *et al*., 2018).

Table 2. Antibacterial activity of Bangladesh-origin Jhum-cultivar *O. basilicum* EO.

Pathogen		Zone of inhibition (mm)[a]			DMSO[b]	Chloramphenicol[c]
		10 µL	15 µL	20 µL		
Gram-positive Bacteria	*S. aureus*	22.7 ± 0.6	24.8 ± 1.0	29.2 ± 0.8	0	25
	B. cereus	33.4 ± 0.8	40.8 ± 1.1	46 ± 1.3	0	28
	Micrococcus spp.	41.8 ± 1.1	46.6 ± 0.5	50.1 ± 1.0	0	25
Gram-negative bacteria	*S.* Typhi	8.7 ± 0.6	9.5 ± 0.5	10 ± 1.0	0	20
	V. cholerae	33.3 ± 0.8	41.3 ± 1.2	45.8 ± 0.8	0	35
	E. coli	8.7 ± 0.6	10 ± 0.0	10 ± 0.0	0	20

[a] Diameter of inhibition zone (mm) around the disc (6 mm); [b] 10 µL 0.5% DMSO was used as negative control; [c] standard antibiotic 10 µg chloramphenicol was used as a positive control.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Bangladesh-origin Jhum-cultivar *O. basilicum* EO.

Pathogen		MIC, mg mL^{-1}	MBC, mg mL^{-1}
Gram-positive Bacteria	*S. aureus*	2.89	5.59
	B. cereus	0.69	1.39
	Micrococcus spp.	2.89	5.59
Gram-negative bacteria	*S.* Typhi	5.59	11.18
	V. cholerae	2.89	5.59
	E. coli	5.59	11.18

Table 4. Comparison of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and major constituents of *O. basilicum* EO from different regions of the world.

Major constituents (%)	Antimicrobial activity		Country		Reference
	MIC	MBC	Pathogen		
Linalool (32.83), Elemol (7.44), Geranyl acetate (6.18)	–	–	–	Algeria	Hadj Khelifa *et al*., 2012
Methyl Chavicol (36.7), Trimethoquinol (10.3), Gitoxigenin (9.3	125, 62.5, 500, 250 (µg mL^{-1})	–	*S. aureus*, *B. cereus*, *E. coli*, *S.* Typhi	Bangladesh (northern Dhaka)	Lira *et al*., 2020
Linalool (48.4), 1,8-Cineole (12.2), Eugenol (6.6)	–	–	–	Egypt	Abou El-Soud *et al*., 2015; Farouk *et al*., 2016
Methyl chavicol (70), Linalyl acetate (22.54)	12.5, 6.25 (mg mL^{-1})	–	*S. aureus*, *E. coli*	India	Sharafati Chaleshtori *et al*., 2018; Srivastava *et al*., 2014
Methyl Chavicol (40.5), Geranial (27.6), Neral (18.5)	18, 18, 9 (µg mL^{-1})	–	*S. aureus*, *B. cereus*, *E. coli*	Iran	Moghaddam *et al*., 2011; Sajjadi, 2006
Linalool (31.6), Methyl chavicol (23.8), *β*-Elemene (6.9)	4.5 µg mL^{-1}	5.5, 6.6 (µg mL^{-1})	*S. aureus*, *E. coli*	Serbia	Sokovic *et al*., 2008; Stanojevic *et al*., 2017
Linalool (61.7), 1,8-Cineole (17.2), Borneol (8.5)	Resistant, 5, 2.5 (mg mL^{-1})	–	*S. aureus*, *B. cereus*, *E. coli*	Nigeria	Usman *et al*., 2013
Linalool (69.87), Geraniol (9.75), *p*-Allylanisole (6)	Sensitive	–	*S. aureus*, *E. coli*, *B. cereus*	Oman	Al Abbasy *et al*., 2015; Hanif *et al*., 2011
Linalool (60), Cadinol, Epi-*α* (12), *α*-Bergamotene (9.2)	Sensitive	–	*S. aureus*, *E. coli*	Pakistan	Hussain *et al*., 2008
Methyl chavicol (93.4), (E)-*β*-ocimene (2.24)	100, 50, 25,50 (µg mL^{-1})	>200, 100, 50, 50 (µg mL^{-1})	*S. aureus*, *B. cereus*, *E. coli*, *S.* Typhi	Thailand	Bunrathep *et al*., 2007; Phanthong *et al*., 2013
Copaene (25.5), *p*-Menth-2-en-1-ol (7.7), Eugenyl acetate (4.8)	Sensitive	–	*S. aureus*, *E. coli*	Ethiopia	Unnithan *et al*., 2013
Linalool (65.38), Eugenol (5.26)	Sensitive	–	*S. aureus*, *E. coli*	Romania	Stefan *et al*., 2013
Linalool (57.08), , *α*-Bergamotene (2.27–	1≤ mg mL^{-1}	–	*S. aureus*	Turkey	Daneshian *et al*., 2009

Major constituents (%)	Antimicrobial activity MIC	MBC	Country Pathogen		Reference
3.70), Naphthalene (11–14.6)					
Methyl eugenol (52.60), Caryophyllene (18.75), Germacrene-D (9.19)	–	–	–	Indonesia	Ni Putu Ermi Hikmawanti, 2019
α-Terpineol (59.78), *β*-caryophyllene (10.54), *α*-humulene (3.90)	–	–	–	Burkina Faso	Bayala *et al*., 2014
Linalool (37), Eugenol(6.3), Geraniol (8.9)	Sensitive	–	*S. aureus*, *E. coli*	Nepal	Yonzon *et al*., 2005
Geranial (35.5), Cis-citral (26.19), Cyclopentanol (5.01)	2.89, 0.69, 2.89, 5.59, 2.89, 5.59 (mg mL^{-1})	5.59, 1.39, 5.59, 11.18, 5.59, 11.18 (mg mL^{-1})	*S. aureus*, *B. cereus*, *Micrococcus* spp., *E. coli*, *V. cholerae*, *S.* Typhi	Bangladesh (Jhum-cultivar)	Present work

'–'= No information

3.3. Antioxidant Activity

The antioxidant activity of Jhum-cultivar *O. basilicum* EO is shown in Table 5. The EO removed 45.70 ± 0.041% free radical at a concentration of 2.39 mg mL^{-1} attributable to the EO constituents' antioxidant properties. Similar levels of inhibition (45.0±0.008) were shown by reference antioxidant ascorbic acid at a concentration of 0.002 mg mL^{-1}. Both the EO and the ascorbic acid showed antiradical activity in a concentration dependent manner. Geranial and cis-citral were the major constituents in the Jhum-cultivar *O. basilicum* EO. Citral isomers, such as trans-citral, cis-citral, and geraniol, have potential antioxidant activity compared to the Trollox standard (Jaradat *et al*., 2016). Besides, citral is a combination of neral and geranial, and both constituents were present in the Jhum-cultivar *O. basilicum* EO. The *O. basilicum* EO has shown antioxidant activity closer to the Serbian variety (2.38±0.01 mg mL^{-1}, 90 min of incubation) (Stanojevic *et al*., 2017). Egyptian *O. basilicum* EO also exhibited a significantly higher scavenging ability for DPPH (Farouk *et al*., 2016).

Table 5. Scavenging effect (%) of positive control and *O. basilicum* EO of Bangladesh-origin Jhum-cultivar on DPPH at different concentrations.

Samples	EO concentration (mg mL^{-1})	Antiradical activities (%)
Essential oil	1.19	29.65±0.011
	2.39	45.70±0.041
Ascorbic acid	0.0013	29.68±0.009
	0.002	45.0±0.008

4. CONCLUSION

The Bangladesh-origin Jhum-cultivar *O. basilicum* EO was studied to explore the bioactive contents plus antibacterial and antioxidant activities. The Jhum-cultivar *O. basilicum* EO confirmed the impacts of origin diversity on EO's composition and the antibacterial and antioxidant response pattern. Therefore, additional studies are required to explore the potential

of Jhum-cultivar *O. basilicum* EO as a source of natural antioxidants for use in the food or pharmaceutical sectors of Bangladesh.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Suman Barua: Conception, Design, Supervision, Fundings, Analysis and/or Interpretation, Writing. **Kajalika Dewan**: Data Collection and/or Processing, Analysis and/or Interpretation, Literature Review. **Saiful Islam**: Data Collection and/or Processing, Writing. **Suman Mojumder**: Data Collection and/or Processing, Writing. **Ovi Sikder**: Data Collection and/or Processing, Writing. **Rajib Sarkar**: Data Collection and/or Processing, Writing. **Hiroshi Hasegawa:** Analysis and/or Interpretation, Critical Review. **Ismail M.M. Rahman:** Conception, Supervision, Fundings, Analysis and/or Interpretation, Critical Review.

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Appendix A. Supplementary information

Figure S1. Antibacterial activity (maximum zone of inhibition) of Bangladesh-origin Jhum-cultivar *O. basilicum* EO against *Micrococcus* spp.

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Research Article

Inula viscosa L. (Asteraceae): A study on its antimicrobial and antioxidant activities, chromatographic fingerprinting profile

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Abstract: Food products contaminated with pathogens and spoiled not only lead to a decrease in the quality and quantity of food products but also contribute to the spread of diseases, which are increasingly becoming a public health problem in both developed and developing countries. Due to the multiple resistance of these pathogens to antibiotics, the search for natural products with antimicrobial properties is becoming increasingly important. *Inula viscosa* has been used as a medicinal plant for a long time in many Mediterranean countries. The aim of this study was to investigate the antimicrobial effects of *I. viscosa* extracts against foodborne pathogens and their non-enzymatic antioxidant potential. Antimicrobial activity was measured using the disc diffusion method. Additionally, plant extracts were tested against 2,2-Diphenyl-1-picrylhydrazyl and 2,2'-azinobis (3-ethyl-benzothiazoline 6-sulfonate) radicals for antioxidant activity. *Inula viscosa* showed the highest antibacterial activity against *Bacillus subtilis* with the methanol extract (19 mm zone diameter), while the lowest activity was observed against *Salmonella* Typhimurium, with inhibition zone diameters of 7 mm. The highest antioxidant activity was recorded as 77.5% for the DPPH• method and 73.8% for the ABTS• method. In conclusion, this plant can be considered a natural antimicrobial and antioxidant agent against foodborne pathogens, and it is a promising candidate for large-scale experiments.

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1. INTRODUCTION

It is estimated that annual global food losses reach up to 40% due to various factors, including spoilage by microorganisms (Gonelimali *et al*., 2018). Bacteria, yeast, and molds are among the common types of microorganisms responsible for the spoilage of a significant number of food and food products (Lianou *et al*., 2016). Foodborne illnesses are another common food safety problem caused by the consumption of contaminated food products, which is a major safety concern for public health (Kirk *et al*., 2017).

It is known that products obtained from both the above-ground and underground parts of plants are used in traditional medicine in our country (Günter *et al*., 2020). Today, in addition

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to meeting basic needs, bioactive compounds from plants are used in various fields such as the pharmaceutical industry, chemistry, cosmetics, and agricultural control (Birch & Bonwick, 2018; Daliu *et al*., 2018; Aguiar *et al*., 2019; Reque & Brandelli, 2021). Furthermore, due to the side effects of synthetic drugs, especially bacterial resistance to them, the importance of natural plant-derived ingredients has increased even more today (Çolak *et al*., 2020).

The *Inula* L. genus, consisting of nearly 100 species worldwide, is predominantly distributed in the Mediterranean region (Gökbulut *et al*., 2013). *Inula viscosa* L. is a perennial herbaceous species belonging to the Asteraceae family and is known as "kanserotu" in Türkiye (Mohti *et al*., 2020). This plant has been traditionally used for its anti-inflammatory, antipyretic, and antiseptic properties (Özhan *et al*., 2013), as well as in the treatment of diabetes, cancer, hypertension, bronchitis, tuberculosis, and various other disorders in traditional medicine for many years (Fontana *et al*., 2007; Ozkan *et al*., 2019).

In this study, the aim was to determine the antioxidant activity as well as the antimicrobial activity of *Inula viscosa* against food-spoilage pathogens.

2. MATERIAL and METHODS

2.1. Plant Material

Inula viscosa was collected from the C2 region (Mugla) of Türkiye (37°01'29.40"N; 28°30'13.79"E) in 2020. The plant material was identified by Dr. Olcay Ceylan, and the plant is stored in the herbarium of Mugla Sitki Kocman University, Department of Biology, Türkiye. The plant was identified according to Flora of Turkey (Davis (1965-1988; Grierson, 1975).

2.2. Preparation of Plant Material

The samples were washed 2-3 times in running water and once in sterile distilled water. Plant parts were air-dried and then powdered using a shredder. All materials were stored at room temperature until sample preparation, and then they were kept at 4°C until needed for analysis.

2.3. Plant Extraction

The air-dried and powdered samples were extracted with water, methanol, and ethanol using a Soxhlet apparatus. After the extracts in organic solvents were evaporated, they were preserved in their respective solvents in small, sterile, opaque bottles and stored in a refrigerator until they were needed.

2.4. Microorganisms

There are 6 foodborne pathogens used in the study, all of which are bacteria. These bacteria include *Bacillus subtilis* RSKK245, *Staphylococcus aureus* RSKK2392, *Salmonella* Typhimurium RSKK19, *Enterococcus faecalis* ATCC8093, *Yersinia enterocolitica* NCTC11174, and *Listeria monocytogenes* ATCC644. Bacterial cultures were grown in Mueller-Hinton Broth (Merck) medium for 24 hours at 37°C. The microorganisms were obtained from the American Type Culture Collection, National Collection of Type Cultures, and the Refik Saydam National Culture Collection.

2.5. Cultivation

Microorganisms were grown in Mueller-Hinton Broth (MHB, Merck) medium by incubating them at their respective temperatures for 24 hours. Active cultures grown for 18 hours were used for all experiments. The turbidity of all microbial cultures was adjusted to 0.5 McFarland.

2.6. Determination of in vitro Antimicrobial Activity

Antimicrobial activity studies were performed using the disk diffusion method (Bauer & Kirby, 1966). Plant extracts (400 mg/ml) were tested by the disk diffusion method, and cultures were

incubated on Mueller-Hinton Agar plates (MHA, Merck) for 24 hours at their respective temperatures. The turbidity of bacterial cultures was adjusted to 0.5 McFarland. Chloramphenicol (30 µg) antibiotic was used as a positive control.

2.7. Determination of the Minimum Inhibitory Concentration (MIC)

The broth dilution method was tested as defined in the CLSI standards (CLSI, 2003; CLSI, 2006). This test was adjusted for the final concentrations of each extract, which were 13.000; 6.500; 3.250; 1.625; and 812.5 µg/mL.

2.8. Determination of Non-Enzymatic Antioxidant Activity

In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH*) and 2,2'-azino-bis (3 ethyl benzo thiazoline-6-sulfonic acid) (ABTS*) radical scavenger activities were used to assess the antioxidant activities of plant extracts (Brand-Williams *et al*., 1995; Re *et al.*, 1999). The results of the trials are reported in mM trolox (TE) per milligram of dry weight.

2.9. Thin Layer Chromatography Analysis for Components of Plant Extracts

This assay was conducted using thin-layer chromatography (TLC) on aluminum sheets coated with silica gel 60F254 (Merck). The extracts (5 spots) were applied to TLC plates and run in a chloroform:methanol mixture (4.5:0.5, v/v). Each plant extract was spotted onto a plate using volumetric micropipettes, along a virtual line positioned 50 mm from the bottom edge of the plate (6.7 x 0.4 cm). The spots were applied at 10-mm intervals. After the chromatogram was developed, the plates were dried, and the spots were visualized sequentially (Zingales, 1967; Zingales, 1968).

3. RESULTS

In our study, which examined the antimicrobial activities of *Inula viscosa* extracts against foodborne pathogens, the zone diameters observed against the tested microorganisms are presented in Table 1. The highest activity was achieved with the methanol extract against *Bacillus subtilis* RSKK245, resulting in a zone diameter of 19 mm (Table 1).

Table 1. Antimicrobial activities of *Inula viscosa* against food pathogens (400 mg/ml)

Microorganisms	Inhibition zone diameter (mm)						
	EE	ME	AE	E	M	A	C
Bacillus subtilis RSKK245	16	19	17	-	-	-	12
Staphylococcus aureus RSKK2392	9	12	8	-	-	-	15
Salmonella Typhimurium RSKK19	8	8	7	-	-	-	22
Enterococcus faecalis ATCC8093	-	8	-	-	-	-	22
Listeria monocytogenes ATCC7644	9	10	8	-	-	-	22
Yersinia enterocolitica NCTC11174	8	9	-	-	-	-	20

EE: Ethanol Extract; ME: Methanol Extract; AE: Aqueous Extract; E: Ethanol; M: Methanol; A: Water; C: Chloramphenicol; (-): No inhibition

Table 2 contains the MIC values of the various solvents obtained from the broth dilution method for the plant used in the study. The lowest MIC value, 3250 µg/mL, was determined for the methanol extract of *Inula* against *Bacillus subtilis* RSKK245 (Table 2).

Table 2. Minimum inhibitory concentrations of *Inula viscosa* against food pathogens (µg/ml)

Microorganisms	Minimum inhibitory concentration		
	EE	ME	AE
Bacillus subtilis RSKK245	13000	3250	6500
Staphylococcus aureus RSKK2392	13000	6500	_
Salmonella Typhimurium RSKK19	13000	13000	_
Enterococcus faecalis ATCC8093	NT	13000	NT
Listeria monocytogenes ATCC7644	13000	6500	_
Yersinia enterocolitica NCTC11174	_	13000	NT

EE: Ethanol Extract; ME: Methanol Extract; AE: Aqueous Extract; NT: not tested; (-): No activity could be detected at values up to 13000 µg/ml

The antioxidant activities of *Inula viscosa* were determined using the DPPH and ABTS methods. In this study, the radical scavenging activity of the ethanol extract was 77.5%, that of the methanol extract was 76%, and that of the aqueous extract was 58.7%. Therefore, the DPPH activity was determined as follows: ethanol > methanol > water (Table 3).

Table 3. DPPH radical scavenging activities of *Inula viscosa*.

Plant (400mg/ml)	EE		ME		AE	
	SA	TE	SA	TE	SA	TE
Inula viscosa	77.5	2.3	76.3	2.3	58.7	2.08

EE: Ethanol Extract; ME: Methanol Extract; AE: Aqueous Extract; SA: Scavenging activity (%); TE: Trolox equivalent (mM trolox (TE)/mg dry weight)

When analyzing the ABTS radical scavenging activity data, which is another antioxidant analysis, the ethanol, methanol, and water extracts of *Inula viscosa* were found to be 72%, 71.1%, and 73.8%, respectively (Table 4).

Table 4. ABTS radical scavenging activities of *Inula viscosa*.

Plant (400mg/ml)	EE		ME		AE	
	SA	TE	SA	TE	SA	TE
Inula viscosa	72.3	2.18	71.1	2.15	73.8	2.2

EE: Ethanol Extract; ME: Methanol Extract; AE: Aqueous Extract; SA: Scavenging activity (%); TE: Trolox equivalent (mM trolox (TE)/mg dry weight)

Thin-layer chromatography is usually conducted for a better identification of bioactive compounds. In the present study, the plates were run in chloroform:methanol (4.5:0.5) and developed with E, M, and S, which indicate ethanol, methanol, and aqueous extracts, respectively (Figure 1). TLC studies revealed the presence of at least 5 compounds in the methanol, ethanol, and aqueous extracts. The three components were separated in the ethanol extract of the plant, while only one component was separated in the methanol and water extracts of *Inula viscosa*. It was observed that among the three solvents, ethanol was the most effective in extracting the maximum number of secondary metabolites (Figure 2).

Figure 1. Thin-layer-chromatography of extracts made from *Inula viscosa*

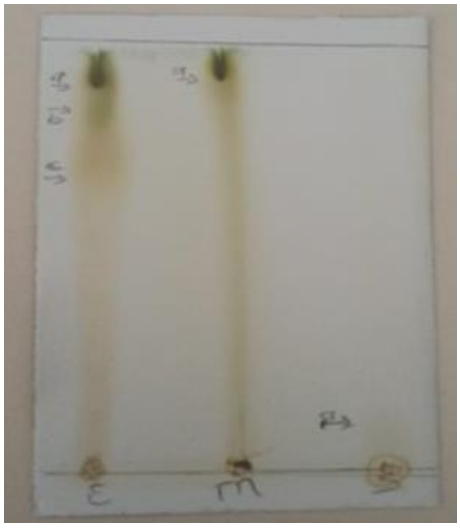
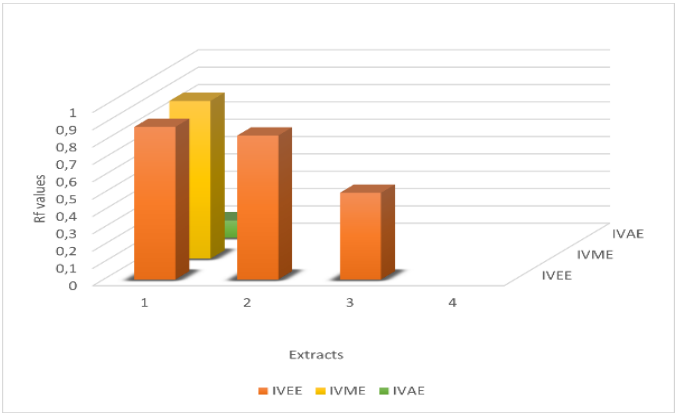


Figure 2. The Rf values of components of *Inula viscosa*



IVEE: Ethanol extract, IVME: Methanol extract, IVAE: Aquoeus extract of *Inula viscosa*

4. DISCUSSION and CONCLUSION

Najefi *et al*. (2011) examined the antimicrobial activities of the phenolic and non-phenolic fractions of *Inula viscosa*. In their study, they tested these fractions against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Salmonella* Entritidis. The zone diameters of the total extract (480 mg/ml) against these bacteria were 15 mm, 19 mm, and 17 mm, respectively. Ozkan *et al.* (2019) tested *Inula viscosa* for antimicrobial activity, using 10 different bacteria in their study. According to the study, the inhibition zone diameter of the methanol extract was 12 mm for *Staphylococcus aureus*. In another study, different extracts of *Citrus aurantium* flowers were tested against various bacteria, including *Listeria monocytogenes*, *Salmonella aureus*, *Bacillus cereus*, and *S.* Typhimurium. The zone diameters for these bacteria were 22 mm, 24 mm, 26 mm, and 16 mm, respectively (Değirmenci and Erkurt, 2020). Tomar and Yıldırım (2019) investigated the antimicrobial and antioxidant activities of *Beta vulgaris* against some foodborne pathogens. Researchers reported that the water extract exhibited high antibacterial activity against *Listeria monocytogenes* (17 mm zone). The data in the literature support our study.

Mohti *et al*. (2020) reported the antimicrobial, antioxidant, and phenolic compounds of *Inula viscosa*. The researchers found that the MIC value against bacteria was 250 µg/ml. In another study, researchers investigated three *Inula* species and tested them against six different

microorganisms. According to the findings obtained at the end of the study, it was reported that the MIC values of *Inula viscosa* varied between 50 µg/ml and 800 µg/ml against these bacteria (Gökbulut *et al*., 2013). Sassi *et al*. (2007) reported the antimicrobial and antioxidant potentials of some medicinal plants collected from Tunisia. Looking at the results obtained in the study, they found that the MIC value of *Inula viscosa* methanol extract was 625 µg/ml for *Staphylococcus aureus*. Talib *et al*. (2012) examined compounds isolated from *Inula viscosa* for their antimicrobial effects. In this study, the plant did not show antibacterial activity against *S. aureus*. However, the plant was effective against other bacteria and yeast. As can be understood from all the data provided in the literature, these studies are in accordance with the data we obtained in our study and support them.

Researchers have reported that the antioxidant activity of *Inula viscosa* has different IC_{50} values (Danino *et al*., 2009; Chahmi *et al*., 2015; Mahmoudi *et al*., 2016; Salim *et al*., 2017; Kheyar-Kraouche *et al.,* 2018). Mitic *et al.* (2020) researched the antioxidant activity of *Inula oculuschristi* in a study. When examining the data they obtained at the end of the study, they reported that the radical scavenging activities of DPPH and ABTS were 57% and 82.7%, respectively. When the data obtained from the literature are compared with our study, it can be seen that the results support our analysis. Additionally, pharmacologically active compounds found in *I. viscosa* include phenolic acids, terpenes, and glycolipids (Fontana *et al*., 2007; Danino *et al*., 2009; Karamenderes & Zeybek, 2000; Andolfi *et al*., 2013). Hispidulin is a naturally occurring flavone present in several traditional Chinese medicinal herbs, including *I. viscosa* (He *et al*., 2011; Xie *et al*., 2015). These compounds discovered in *Inula* also explain the high antioxidant activity detected in our study.

Different Rf values of the compounds provide an idea about their polarity, which may also help in selecting a particular solvent system for further isolation of any compound from the plant extracts using chromatographic and spectroscopic techniques (Biradar & Rachetti, 2013). Compounds showing a high Rf value in the less polar solvent system have low polarity, while those with a low Rf value have high polarity (Talukdar *et al.*, 2010). In this study, 5 bioactive components with different Rf values were determined. These components have varying polarities (Figure 2).

The public health problem caused by microorganisms in foods has made this issue very significant. Antibiotics are used to eliminate and combat bacteria in foods, but their use can lead to bacteria developing resistance. This has highlighted the importance of focusing on naturally available plant-derived substances that bacteria cannot develop resistance to and that naturally inhibit or kill them.

In this study, the effectiveness of the *Inula* plant against these microorganisms was determined, and it is believed to be a strong candidate for use in food preservation due to its high antimicrobial and antioxidant activity. The present study confirms the presence of many important phytochemicals in *Inula viscosa* from Turkey. Observing the results of the chromatography analysis, it can be concluded that *Inula* produces many secondary metabolites of medicinal value. Therefore, the plant can be used as a source to produce phytochemicals using advanced techniques of extraction, screening, identification, and isolation. To consider the use of this product in phytotherapy, we need to further determine the composition of the aerial parts of the plant.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflicts of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). .

Authorship Contribution Statement

Gulten Okmen: Conception, Design, Supervision, Fundings, Analysis and/or Interpretation, Writing, Critical Review. **Kutbettin Arsalan**: Materials, Data Collection and/or Processing, Literature Review. **Ridvan Tekin**: Materials, Data Collection and/or Processing, Literature Review.

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Research Article

Aroma profile of the essential oils from different parts of *Pycnocycla aucherana* Decne. ex Boiss.

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Abstract: The current study focused on the essential oil concentration and aroma profile in different parts of wild *Pycnocycla aucherana* Decne from Iran during two years 2017 and 2018. Plant samples of *P. aucherana* were collected from HajiAbad area located in Hormozgan province, Iran at reproductive stage on June 7, 2017 and 2018. Essential oil isolation was done by hydro-distillation method for 3 hours. For the qualification and quantification of components, gas chromatography/mass spectrometry (GC/MS) was applied. Results showed that the averages of essential oil percentage of shoot and leaf (SL) and seed in the first and second year were (0.13, 0.23%) and (0.4, 0.3%) respectively. The main chemotype was namely α-phellandrene (5.96-16%), *p*-cymene (3.07-27.4%), Limonene (0.72-6.80%), γ-cadinene (0.8-4.33%), Spathulenol (1.90-8.64%), Elemol (0.3-6.69%), β-eudesmol (0.8-9.27), and Bulnesol (0.91-3.40%). The highest amount of α-phellandrene (16%) and *p*-cymene (27.4%) was observed in the seed and (SL) of essential oils in the first year respectively. Elemol and Elemicin content increased during the second year in the seed of essential oils with amount of (6.69 %) and (25.69 %), respectively. Overall, the results showed that the geographic origin greatly influenced the chemical composition of *P. aucherana*.

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Citrus sinesis, antimicrobial activity, antioxidant activity, Molecular docking, Essential oils.

1. INTRODUCTION

Native medicinal plants play an important role in providing many food and medicine needs for local people in each region (Emami Bistgani & Sefidkon, 2019; Petropoulos *et al.*, 2020). Collecting endemic medicinal and range plants is one of the traditional cultures which have been conserved in numerous indigenous societies (Ghasemi *et al.*, 2013; Janaćković *et al.*, 2019). Endemic medicinal and range plants were gathered for different goals. Wild plants were

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usually consumed to provide our predecessor' substantial requirements comprising silage, food, fuel wood, and production supplies (Fallah *et al.*, 2020). These kind of collected herbs comprise favorite aroma profiles which function in pharmaceutical and cosmetic industries (Debbabi *et al.*, 2020). Prior research has revealed that wild medicinal and range plants contain upper bioactive compounds and pharmacological activity when compared to cultivated plants. For instance, a study conducted by Jung *et al.* (2005) indicated that the leaves of planted ginseng in the field have lower natural components than those of wild ginseng leaves. In addition, Soriano-Melgar *et al.*, (2012) indicated that domain as a wild shrub comprises greater antioxidant properties than the cultivated of that.

Pynocycla genus belongs to the Apiaceae family which is endemic to tropical areas. In Iran, it grows in Kermanshah, Khuzestan, Fars, Kerman, Hormozgan as well as Systan and Baluchestan provinces (Central and South parts of Iran) (Mozaffarian, 1996), *Pycnocycla* species are perennial and woody plants with multiple erect stem (Mozaffarian, 2007). Because of the aromatic nature of *Pycnocycla* species, different reports are available about chemical composition of the essential oil (Asgarpanah *et al.*, 2014; Asghari *et al.*, 2014; Sadraei *et al.*, 2016). Some studies conducted by Teimouri *et al.* (2005) and Alimirzaloo and Asgarpanah, (2017) indicated that □-phellandrene and p-cymene were the main compounds in the essential oil of the leaves in *Pycnocyla aucherana*. However, trans-isomyristicin was identified as the major component in the seed. Generally, diverse pharmacological activity of *P. aucherana* including antispasmodic (Sadraei *et al.*, 2016), anti-inflammatory (Jahandar *et al.*, 2018), and cytotoxic effects (Khodaei, 2012) have been reported by researchers.

As a matter of fact, the essential oil of the genus of *Pycnocycla* content that has effective aroma profiles and plays a significant role in pharmacological activities is ultimately used in the cosmetic and health industries (Askari *et al.*, 2022). For instance, terpenoids represent the most diverse and largest class of chemicals among the secondary metabolites produced by higher plants (Tholl, 2015). Monoterpenes and sesquiterpenes are as the main volatile constituents, which provide characteristic aroma and biological properties to the essential oils and are important flavoring and fragrance agents in food products, beverages, cosmetics, and pharmaceuticals (Askari *et al.*, 2022).

Up to now, limited studies have reported the aroma profile of *Pycnocycla aucherana* in Hormozgan province that is rich in essential oil (i.e., high yields) and phenolic monoterpenes (Askari *et al.*, 2022). Therefore, the aim of this research was to determine the phenolic profiling in shoot and leaf (SL) as well as seed in *P. aucherana* collected from natural habitats of Hormozgan province during two years (2017 and 2018).

2. MATERIAL and METHODS

2.1. Plant Materials

Plant samples of *P. aucherana* were collected from HajiAbad area located in Hormozgan province, Iran at reproductive stage on June 7, 2017 and 2018. Then the herbs were deposited in the herbarium of Research Institute of Forests and Rangelands, Tehran. The herbarium code number was TARI 5828. The shoot and leaf (SL) as well as seed were separated and dried at room temperature. 100 g of (SL) and 80 g seed were used for essential oil extraction. Essential oil isolation was done by hydro-distillation method and Clevenger-type apparatus for 3 hours. The essential oils were dried over anhydrous sodium sulfate and stored in sealed vials at 4 ^{o}C before analysis.

2.2. Analysis of The Essential Oil

To determination quantity and quality of the essential oil, gas chromatography (GC) and gas chromatograph-mass spectrometry (GC/MS) were applied in Chromatography Analysis

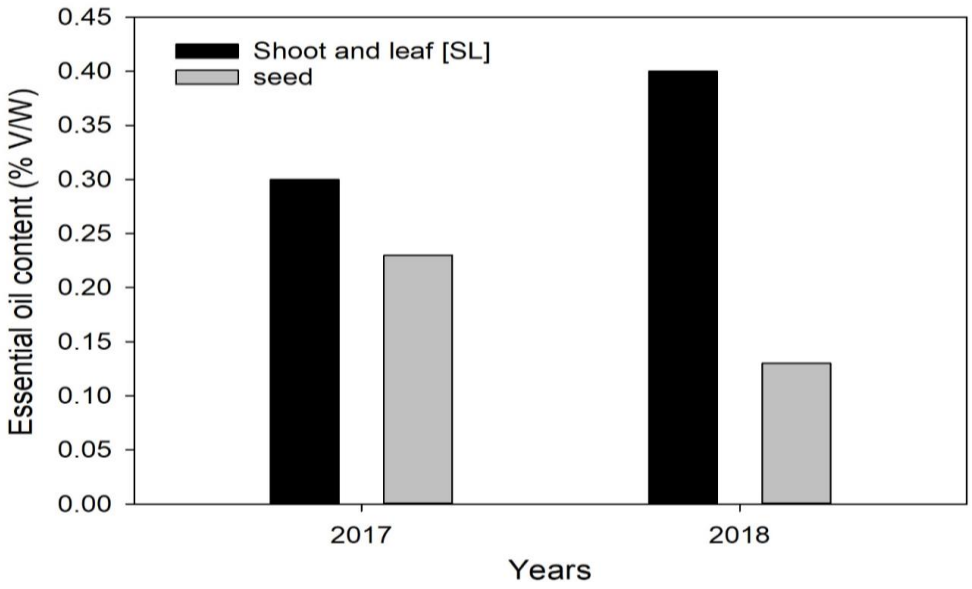
Laboratory of Medicinal plants and by-products Research department. To determine quantification of the essential oils, Ultra Gas chromatograph equipped with a DB-5 (Thermo model UFM) fused silica column (60 m x 0.25mm, film thickness 0.25 micron) was used. The temperature in oven was set on 50–270 °C at a flow rate of 40 °C /min and then detained at 280°C for 3 min. Injector and detector temperatures were 280 °C; a linear velocity of 32 cm/s was set out for the helium gas. GC–MS analyses were performed (Varian 3400 GC–MS system, Varian, Palo Alto, CA, USA) on DB-5 (60 m x 0.25mm, film thickness 0.25 micron). The oven temperature was set on 60–240 °C at a rate of 3 °C /min. The transfer and injector line temperatures were 260 and 240 °C, respectively. The carrier gas was helium at linear velocity of 32 cm/s. The split ratio was adjusted in 1:60, and in mode at ionization energy of 70 eV and a scan time 1. The Kovats retention index (RI) of constituents was calculated by their mass spectra with those of authentic compounds of n-alkanes (C_6–C_{24}) (Adams 2017).

3. RESULTS

3.1. Essential Oil Concentration In Different Parts of *Pycnocycla Aucherana* Decne

The essential oil extracted by Clevenger-type apparatus from the seed was colorless, while the essoil of shoot and leaf was pale yellow in *P. aucherana*. According to the obtained results, the averaential ge essential oil percentage of (SL) in the first and second year was 0.13 and 0.23%, respectively. On the other hand, the average essential oil percentage of seed was 0.4 and 0.3% in first and second year, respectively (Figure 1).

Figure 1. Essential oil concentration of shoot and leaf (SL) and seed in *Pycnocycla aucherana* Decne



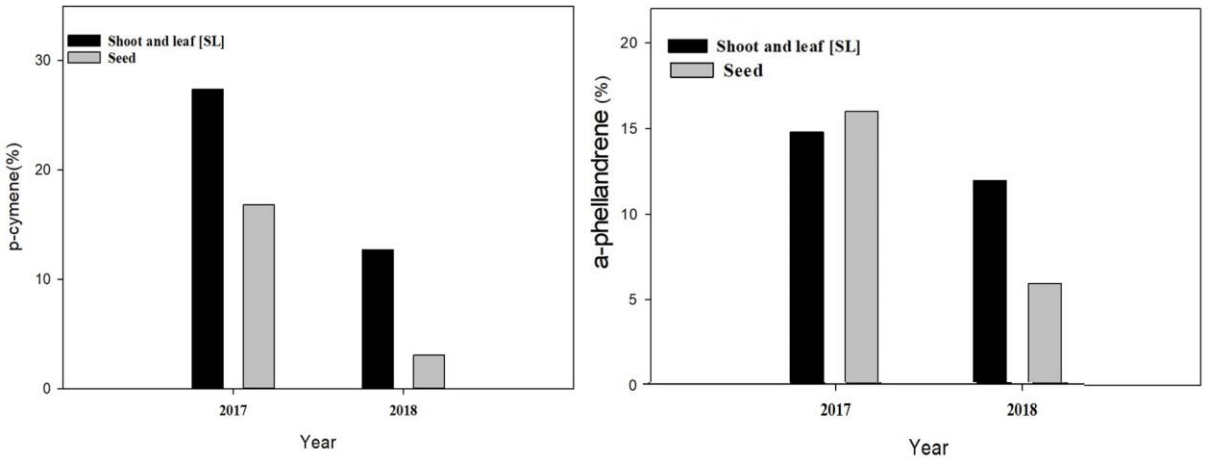
3.2. Volatile Profile of The Essential Oil in Different Parts of *Pycnocycla Aucherana*

Volatile profile of the essential oil obtained from the SL as well as seed of *Pycnocycla aucherana* is presented in Table 1. 22 to 28 components were identified in different samples including (SL and seed) which consisted of (86.31-98.3%) in the essential oils. In the essential oils of SL 22-26, chemical constituents recognized were approximately (91.62 -93.2%). In the essential oil of the seed, 27-28 chemical compounds were identified which present around (86.31- 98.3%) of total essential oil.

The main chemical compounds included α-phellandrene (5.96-16%), *p*-cymene (3.07-27.4%), Limonene (0.72-6.80%), γ-cadinene (0.8-4.33%), Spathulenol (1.90-8.64%), Elemol (0.3-6.69%), β-eudesmol (0.8-9.27), Bulnesol (0.91-3.40%) in SL and seed essential oils during

two consecutive years. The highest concentration of aroma profiles was allocated to *p*-cymene as the main aromatic organic compound in the SL in the first harvesting. The concentration of *p*-cymene fluctuated between (27.4-12.72%) in the essential oil of SL and (3.07-16.8%) in the essential oil of seeds (Figure 2).

Figure 2. *p*-cymene and α-phellandrene content of different part of the *P.aucherana*.



The percentage of α-phellandrene varied between (11.97-14.8%) in the essential oil of SL and (5.96-16%) in the essential oil of seed (Figure 2). The range of limonene as one of the important natural compounds varied between (0.72-5.50%) and (2.02-6.80%) in the seeds and SL in both years, respectively. In addition, Spathulenol as the tricyclic sesquiterpene alcohol varied between (1.9- 3.57%) and (2.20-8.64%) in the seed and SL, respectively.

Trimethylbenzaldehyde was detected in the essential oils of SL and seed in the second year of the harvested plants with amount of 13.32% and 5.84%, respectively. The percentage of Elemicin in SL and seed oil was 2.81% and 25.89% in the second year of the experiment, respectively. It should be noted that Sylvestrene (3.6, 2.7%), Terpinen-4-ol (3.9, 1.2%), linalool acetate (3.0, 0.9%), Sesquisabinene hydrate (5.4, 1.9%), Caryophyllene oxide (4.7, 2.3%) and β-bisabolol (8.8, 2.6%) were identified in SL and seed respectively in the first year of harvested plants. On the other hand, 1,8-cineole (3.55, 1.70%), Trimethylbenzaldehyde (13.32, 5.87%), δ-curcumene (2.86, 7.66%), Elemicin (2,81, 25.89%), and Guaiol (7.26, 7.71%) were observed in SL and seed respectively in the second year of harvesting plants. It is interesting to note that E-isoelemicin with amount of (26.2 %) was only observed in the seed of essential oils in the first year. Overall, it seems that changes in concentration were not almost the same trends in both years of experiment. It might be that the fluctuations of temperature and moisture were strong enough to stimulate the high concentration of chemical variability recorded. The difference in aroma profile in two years exhibited by the species was also to be related to the climatic conditions of the sampling area, as the plant was growing at a different altitude.

Table 1. Aroma profile in different parts of *P. aucherana* during two consecutive years.

			2017		2018	
Number	Aroma profile	RI	Shoot and leaf [SL]	Seed	Shoot and leaf [SL]	Seed
1	α-thujene	924	-	0.20	-	-
2	α-pinene	932	0.60	1.0	-	-
3	Sabinene	969	-	0.30	-	0.26
4	β-pinene	974	0.40	1.0	0.65	1.25
5	Myrcene	991	0.20	7.6	0.92	0.20
6	Octanal	998	-	0.30	-	-
7	α-phellandrene	1002	14.8	16.0	11.97	5.96
8	*p*-cymene	1020	27.4	16.8	12.72	3.07
9	Limonene	1024	6.80	5.50	2.02	0.72
10	Sylvestrene	1025	3.60	2.70	-	-
11	1,8-cineole	1026	-	-	3.55	1.70
12	Linalool	1095	-	0.50	-	0.18
13	Trans-pinocarveol	1135	-	-	1.11	0.48
14	Terpinen-4-ol	1174	3.90	1.20	-	-
15	Cryptone	1183	0.80	0.30	-	-
16	Citronellol	1223	0.40	-	-	-
17	linalool acetate	1254	3.0	0.90	-	-
18	(E-2) decanal	1260	-	-	0.38	0.44
19	Citronelly formate	1271	0.30	-	-	-
20	Bornyl acetate	1284	0.20	-	-	-
21	Thymol	1289	-	-	0.32	1.15
22	Trans-pinocarvyl acetate	1298	-	-	-	0.28
23	Carvacrol	1298	-	-	1.65	0.39
24	Terpinene-4-ol-acetate	1299	0.20	-	-	-
25	Trimethylbenzaldehyde	1313	-	-	13.32	5.84
26	Isoledene	1374	0.30	0.50	-	-
27	Methyl eugenol	1403	-	0.70	-	-
28	E-caryophyllene	1417	0.40	0.70	0.81	1.82
29	Aromadendrene	1439	-	-	-	0.48
30	α-humulene	1452	-	-	-	0.59
31	γ-munrolene	1478	0.50	2.30	-	-
32	δ-curcumene	1481	-	-	2.86	7.66
33	Germacrene D	1484	-	-	0.34	0.60
34	Bicyclogermacrene	1500	-	-	2.54	2.83
35	α-bulnesene	1509	0.70	0.80	-	-
36	γ-cadinene	1513	0.80	0.80	2.22	4.33
37	Sesquisabinene hydrate	1542	5.40	1.90	-	-
38	Elemicin	1555	-	-	2.81	25.89
39	Spathulenol	1577	2.20	1.90	8.64	3.57
40	Caryophyllene oxide	1582	4.70	2.30	-	-
41	Elemol	1584	0.70	0.30	4.72	6.69
42	E-isoelemicin	1586	-	26.2	-	-
43	Guaiol	1600	-	-	7.26	7.71
44	β-eudesmol	1649	2.7	0.80	9.27	7.80
45	Bulnesol	1670	3.40	2.20	0.91	1.75
46	β-bisabolol	1674	8.80	2.60	-	-
47	Total		93.2	98.3	91.62	86.31

4. DISCUSSION and CONCLUSION

Based on the results of evaluating the essential oil percentage which is extracted from various organs, the highest essential oil was obtained in the seed with amount of 0.40% and the lowest essential oil concentration was seen in SL with amount of 0.13% in the first year. Therefore, it seems that seed essential oil in the first year was approximately 25% more than that of the second year. The results obtained from this study were either in accordance or opposite with previous studies. For instance, essential oil content of various *Pycnocyla* species varied between 0.05%-3.8% (Alimirzaloo & Asgarpanah, 2017; Nasr & Asgarpanah, 2014). The essential oil percentage of fruit and seeds of *P. aucherana* collected from Hormozgan province was between 0.1% and 3.8% (v/w) (Alimirzaloo & Asgarpanah, 2017). Another report presented that essential oil percentage of this species was 0.12 % (Teimouri *et al.*, 2005). It seems that essential oil content varied according to the species, organs, and site of collection (Oueslati *et al.*, 2019). As a matter of fact, essential oil concentration and composition are affected by harvesting time, climate condition as well as plant parts (Emami Bistgani *et al.*, 2017a; Emami Bistghani *et al.*, 2012; Sefidkon & Emami Bistgani, 2021).

These results illustrated that although the production of secondary metabolites such as essential oils has a long evolutionary and genetic background; however, it is possible to observe fluctuations in the amount of essential oil of the aerial parts and seed plants collected from different habitats and areas (Mitić *et al.*, 2018; Delfine *et al.*, 2017).

p-cymene and *α*-phellandrene were attained in SL and seed for two years. The highest *p*-cymene as the phenolic monoterpenoids (27.4 %) was observed in SL sample and the maximum *α*-phellandrene (16%) was detected in the seed sample (Table 1). Interestingly, *p*-cymene and *α*-phellandrene were as the main compounds in *P. aucherana* in reports of (Teimouri *et al.*, 2005; Alimirzaloo & Asgarpanah, 2017). Also, *α*-phellandrene was found in *P. flabellifolia* (Mahboubi *et al.*, 2016).

Trimethyl benzaldehyde was observed in SL (13.32 %) and seed (5.87%) at the second year. This compound was reported in aerial part (13.2 %) of *Pycnocycla caespitosa* (Asgarpanah *et al.*, 2014) which are in agreement with our results in SL sample. Elemicin was also found in SL (2.81%) and seed (25.89 %) in the second year of the experiment. Asghari and Houshfar (2001) identified that Elemicin is the main chemical compound in the *Pycnocycla spinosa* var spinosa. The concentration of Elemicin in *Pycnocycla spinosa* was 60.1% (Mahboubi *et al.*, 2016). Among identified compounds E-isoelimicin with an amount of (26.1%) was only found in seed sample in the first year. Spathulenol (8.64%) and *β*-eudesmol (9.27%) indicated high amount of concentration in SL in the second year. Spathulenol was also reported in *Pycnocycla caespitosa* by (Asgarpanah *et al.*, 2014) and *Pycnocycla nodiflora* (Nasr & Asgarpanah, 2014). *β*-eudesmol was observed in *Pycnocycla caespitosa* (Asgarpanah *et al.*, 2014).

Sefidkon & Emami Bistgani (2021) showed that chemical variation in essential oil can be explained by the following factors: 1) genetic variation in the population; 2) diversity among different parts of each plant and growth stages; 3) environmental factors. Furthermore, it was reported that the medicinal plant substances, especially secondary metabolites, are the reflection of environmental factors, growth stage and plant genetic background (Liu *et al.*, 2015). Weather factors including temperature, precipitation and soil can affect oil content and composition in many aromatic plants (Moghaddam & Farhadi, 2015).

In the most studies it was indicated that essential oil content and chemical composition were influenced by harvest time and climate condition such as *Achillea millefolium* (Aziz *et al.*, 2018) *Ocimum minimum* L. (Figueredo *et al.*, 2017), *Salvia officinalis* (Zawiślak *et al.*, 2014), and *Thymbra spicata* (İnan *et al.*, 2011). Evaluations have shown that in addition to the role of genetic factors and environment in the production and quantitative and qualitative diversity of

the essential oil compounds, other factors such as organs of the plant and the stage of the growth in which the plant is located are very effective. Different organs of aromatic plants have a different capacity for production of essential oils and to achieve maximum performance of essential oil and information from plant organs with high percentage of essential oil is very necessary (Reddy, 2019). This issue can be considered for medicinal plants breeder as a purpose of breeding in the dry matter yield of the organs and also for use in pharmaceutical, food and cosmetic industries (Heidarpour, 2013).

Periodic fluctuations in the composition and function of plant essential oils can be justified by various arguments. Simultaneously as plant developments, cell structure and its textures change and chemical compositions change, all of the items can be affected by chemicals components behavior (Morshedloo *et al.*, 2017; Emami Bistgani *et al.*, 2019; Emami Bistgani *et al.*, 2017b; Emami Bistgani *et al.*, 2018). Differences in the composition of essential oils can be partly due to structures glandular trichomes in the leaves. Volatile plant compounds are produced and stored in the specific glandular to minimize the own toxicity and the existence of higher levels of these compounds make it possible as a defense factor in the herb (Jamzad, 2009).

In conclusion, p-cymene and α-phellandrene were found in SL and seed samples during two years. Concerning the fact that these kinds of aroma have biological activities which are related to type of their structures and their relative percentages, such variation in the chemical composition of the studied species assisted the collection of those with higher qualification bioactive constituents for use in relevant industries. On the other hand, Elemicin, E-isoelemicin, spathulenol and b-eudesmol content varied according to year and plant organ. According to obtained results, variation in main chemical compounds can be due to the effect of various organs and ecological, edaphic and climatic factors. The results also demonstrated that determination of this variation is helpful for identification of new components in the species and other species of this genus. Finally, the *Pycnocycla aucherana* species with high bioactive compounds may be recommended for different applications. For instance, it can provide characteristic aroma and biological properties to the essential oils and are important flavoring and fragrance agents in food products, beverages, cosmetics, and pharmaceuticals.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fatemeh Askari: Design the experiment, extration of essential oil, collected valuable data. **Fatemeh Sefidkon:** Phytochemistry analysis, **Zohreh Emami Bistgani:** Formal analysis and writing the manuscript, **Mohamad-Amin Soltanipour:** Collected the seeds.

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Research Article

Investigation of the effect of using palm kernel powder in bread flour on quality parameters of flour

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Abstract: In this study, the effect of palm kernel powder (PKP), a vegetable waste, on the quality parameters of bread and dough was investigated. For this purpose, farinograph and extensograph values of doughs made from PKP-flour (2.5, 5, 7.5 and 10%) mixed flours were investigated. Weight, volume, specific volume, crust color, inner color and firmness values were investigated in bread made from mixed flour. The results showed that the water absorption, development time, stability, resistance to extension and maximum resistance values of the dough increased with the increase in the amount of PKP. It also decreased in degree of softening, extensibility and energy levels. The change in softening degree and stability values were found to be statistically significant (p<0.05). PKP additive caused a firmer structure in bread samples. It was observed that the weight, volume, specific volume, first-day hardness and third-day hardness values of the bread increased with the increase in PKP. L^* and a^* values of bread crust color and inner color decreased with the addition of PKP. The increase in PKP had a positive effect on bread quality by increasing dough stability and bread volume values.

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1. INTRODUCTION

Wheat is a type of grain in the Graminea (Poaceae) family. The grain consists of three parts; the bran, endosperm and germ (embryo) in the outer layer. It contains components such as water, carbohydrates (starch), proteins, lipids, mineral substances, enzymes, vitamins, amino acids and fatty acids (Elgün & Ertugay, 2002; İlerigiden *et al.*, 2020). Wheat grain contains approximately 10% water, 60-75% starch, 10-18% protein, 1.5% lipid and 1.5% mineral matter (Ashraf, 2014). Wheat growing is carried out in large areas with different climate and soil characteristics. Due to the variety and environmental effects, there are differences between the physical, chemical and technological properties of wheat. Bread wheat, called *Triticum aestivum* L. is the most commonly produced type of wheat. red-colored and hard-grained varieties are considered to be the highest quality bread wheat (Elgün & Ertugay, 2002).

Cereals and their products are among the food groups that have an important place in human nutrition. Especially in our country, the consumption amount is high because flour is a cheap source. In societies where bread is consumed as a basic nutritional element, vitamins, minerals

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and amino acids are added to flour in order to increase the flavor and product variety and to enrich its nutritional value. The distribution of the chemical components of wheat varies according to the morphological parts of the grain. Wheat causes a change in its chemical structure while being processed. When separation processes are performed, the amount of some nutrients decreases. In addition, the applications during the process cause changes in the nutrients themselves. For this reason, in many countries of the world, cereal products, especially bread, are enriched with vitamins and minerals that decrease during the processing and especially grinding of wheat (Karagül & Ercan, 1993; McKevith, 2004).

Nutrition is a fundamental aspect of human life, as it is essential for individuals to consume various food items in sufficient and balanced quantities in order to support their growth, development, and active continuation of life (Traş & Gökçen, 2021). However, in today's world, the disregard for proper dietary conditions has resulted in various eating disorders, primarily obesity and excessive eating (Traş & Gökçen, 2021), which in turn can lead to the emergence of several associated health conditions such as cancer, diabetes, lactose intolerance, digestive difficulties, cardiovascular diseases (Ertaş *et al*., 2019), and even certain neurological (autism and ADHD) and psychological disorders (such as GAPS) (Çıkılı *et al*., 2019).

In recent years, there has been a growing interest in healthy and functional food products aimed at meeting high nutritional demands with minimal consumption, which has led to the utilization of functional and alternative ingredients in many food items. Particularly, the incorporation of substitute ingredients rich in vitamins, minerals, and antioxidants, found naturally in their composition, has gained attention (Demir & Kılınç, 2019). This trend has emerged alongside discussions surrounding the negative effects of food on health, prompting the exploration of healthier and functional food products.

Date (*Phoenix dactylifera* L.) is a food source, building material and traditional folk medicine that has been used by humans since ancient times. In addition to the economic value of this plant, it is important to know its health-related aspects (Aktürk & Işık, 2012). It is a herbal product produced in Egypt. However, it is also cultivated in Middle Eastern countries and arid regions of Southwest Asia and North Africa (Diab & Aboul-Ela, 2012). Date fruits are marketed worldwide as a fruit product with rich nutritional content (Al-Farsi & Lee, 2008). Date seeds have generally not received much attention due to their popularity and lack of commercial application. In the Arab world, for centuries, date beans have been marketed as a decaffeinated beverage and decaffeinated coffee (Baliga *et al*., 2011). Although its biological value is low, it is known that the protein content of the palm kernel is between 7.13-10.36% and the oil content is between 6.32-9.28% (Gabrial *et al*., 1981). While there is 0.2-0.5% oil in the fleshy part of the date, the oil rate in the kernel is between 7.7-9.7%. The kernel is 5.6-14.2% of the weight of the date. There are 14 types of fatty acids in its core. 8 of these are also found in small amounts in the meat part. Unsaturated fatty acids include “palmitoleic”, “oleic”, “linoleic” and “linolenic” acids. The amount of oleic acid in the palm kernel varies between 41.1 and 58.8%. Therefore, they can be used as a source of oleic acid. In addition, the date kernel contains 0.5% potassium and various amounts of aluminum, cadmium, chloride, lead, and sulfur (Al-Shahib & Marshall, 2003).

Palm kernels can be used in different industrial areas. Activated carbon obtained from palm kernel waste is used to clean chromium, a poisonous substance, from liquids (El Nemr *et al*., 2008). Date seeds are also rich in phytohormones. For this reason, the hormonal effects of dates are also used in the beauty industry (Bauza *et al*., 2002).

2. MATERIAL and METHODS

2.1. Material

Bread wheat flour and dates (*Phoenix dactylifera* L.) used in the research were obtained from the Balıkesir bazaar. The palm kernels were removed and ground in a grinder.

2.2. Methods

2.2.1. *Obtaining Flour from Wheat and Date Kernel Samples*

Palm kernel was ground to pass through a sieve no 70, 90% of which was 212 µm, in order to be close to the grain size of the flour used in the experiments (Anonymous, 1999) and stored in glass jars with lids to be used when necessary. The powders were stored in a cool, dry environment with the lid closed, away from sunlight, at room temperature. Then, palm kernel powder (PKP) was added to the wheat flour at the rates of 2.5, 5.0, 7.5 and 10% by displacement. Flour without PKP was used as a control sample.

2.2.2. *Determination of Physical, Chemical and Technological Properties on Flour Samples*

The analyses were performed according to the method of AACC (2000). Moisture analysis in wheat flour and PKP was carried out according to the AACC method 44-19, raw ash analysis was performed according to the AACC 08-01 method. Determination of Wet Gluten and Gluten Index was determined according AACC 38-12 method using Glutomatic-2200 and Glutork 2020 (Perten Instruments AB, Huddinge, Sweden) devices. The falling number value was measured by using AACC 56-81b method (AACC, 1990). Zeleny sedimentation test was carried out according to Özkaya & Kahveci (1990). Delayed sedimentation values determination, different from the Zeleny sedimentation test, was determined by measuring after bromine phenol blue was added and left for 2 hours (Greenaway *et al*., 1965). *L** value on the color scale [(0) black-(100) white] using Hunter Lab Color Quest II Minolta CR-400 (Minolta Camera, Co., Ltd., Osaka Japan) device in flour, PKP, flour-PKP mixture and bread, *a** value [(+) red- (-) green] and *b** values [(+) yellow-(-) blue] were measured (Francis, 1998).

The water holding capacity, dough development time, stability, and degree of softening (12 minutes after the maximum value on the curve) in the control and flour blends were determined according to the AACC 54-21 method used by Brabender Farinograph (Brabender, Duisburg, Germany) device (AACC, 2000). The dough's resistance to extension, dough's maximum resistance, extensibility and energy values were determined by using Brabender Extensograph (Brabender, Duisburg, Germany) according to AACC 54-10 method (AACC, 2000). Both rheological analyses were based on 300 g flour.

2.2.3. *Analysis of Bread Samples*

Bread made from control and blended flours was made by modifying the method given in AACC 10-10 (AACC, 1990). Accordingly, on the basis of 100 g flour; dough mixture was prepared by using 3% yeast, 1.5% salt and water as much as the farinograph removes. Doughs kneaded until the maturation phase (Hobart N50, Canada) were rested at 30 °C at 80-90% relative humidity for 50 minutes and baked at 230°C for 25 minutes. Weight and volume were measured after the bread cooled and they were put into polyethylene bags after 1 hour (Elgün *et al*., 2001). Crust and crumb color was also determined by measuring *L**, *a**, and *b** values with a Hunter color measuring device (Francis, 1998; Rossel *et al.,* 2006). Bread volume and specific volume measurements were made according to Elgün *et al*. (2015).

The baked breads were kept at 20–25°C and 40–45% relative humidity for 3 days, and the moisture changes of the samples were measured every day, and the hardness change was measured twice with a texture analyzer (TA Plus, Lloyd Instruments, UK) on the 1st and 3rd days (Abd Karim *et al.,* 2000).

2.3. Statistical Analysis

The samples were analyzed with 2 replications and the difference between the means was determined using analysis of variance (ANOVA), and the level of difference between samples was made using Duncan's test, one of the Multiple Comparison tests

3. RESULTS

3.1. Flour Blend Analysis

The results of the control sample and flour blends are given in Table 1. Parallel to the increasing PKP ratio, a decrease was detected in the protein ratios of the flour samples. The protein ratio may also vary depending on the type of blended product. The seeds of date varieties contain an average of 6.5% protein, 10.4% oil, 22.0% fiber, 1.1% ash, and 60.0% carbohydrates in dry matter (Sawaya, 1984). Due to the fact that the content of the palm kernel is rich in fiber and carbohydrates, it proportionally decreased the protein ratios in the PKP mixture. The increase in the ash content with the increase in PKP indicates that the flour is rich in minerals (Table 1). In a similar study, they observed that the amount of dietary fiber and ash increased significantly (p<0.05) in muffins to which they added palm seed flour and palm seed flour hydrolysate (Ambigaipalan and Shahidi, 2015). Falling number, gluten, gluten index, sedimentation, and delayed sedimentation tests could not be obtained in flour with PKP addition. Palm kernel powder (PKP), differs from flour derived from wheat or other traditional grains in terms of its composition. Therefore, due to the different gluten content and potential for gluten network formation in PKP-added flours, the desired results cannot be obtained from tests such as falling number, gluten, gluten index, sedimentation, and delayed sedimentation.

The farinograph analysis provides important information for evaluating the gluten structure and strength of flour, as well as the processing properties and quality of dough. The results of the farinograph test are used to assess factors such as dough resistance, extensibility, expansion capacity, water-holding capacity, and overall workability. Some parameters (water absorption, development time, stability and softening degree) of farinograph analysis are shown in Table 2. It is seen that as the PKP value increases in flour mixtures, the dough development time and stability (p<0.05) value increase and the softening value decreases. Iqbal *et al*. (2015) stated that dough development time is affected by the concentration and quantity of wheat protein. Long dough development time is a desirable property for bread. The obtained results show that the addition of PKP has a positive effect on the dough development time. High water holding capacity and stability are the feature required by bakers as it facilitates dough processing (Sahin *et al*., 2019). The increase in the PKP ratio did not significantly affect the water holding capacity and also did not even cause a decrease. In addition, as the PKP ratio increases, the stability also increases, which indicates that the dough rheology is positively affected. It shows good quality flour with its high water holding capacity and low softening degree (Biel *et al*., 2021). The findings obtained in this study also confirm the information given in previous studies.

With the increase in the amount of PKP, an increase in the resistance to extension and maximum resistance values, and a decrease in the energy and extensibility values were observed (Table 2). However, it was determined that this change was not statistically significant (p>0.05). The excess of fiber (22%) in the palm kernel content (Sawaya, 1984) decreased the extensibility and energy values of the dough (Table 2). Similar results have been shown in studies with an increase in meal (fiber) in flour, and a decrease in energy values and extensibility (Bilgiçli *et al*., 2007; Gül, 2007; Erdoğan, 2010).

Table 1. Chemical properties of flour blends[1]

PKP Ratio	Moisture (%)	Ash[2] (%)	Protein[3] (%)	Falling number (s)	Gluten (%)	Gluten index (%)	Sedimentation (cc)	Delayed sedimentation (cc)
Control[4]	9.84±0.03[b]	0.44±0.05	10.95±0.3	352±21.5	27±0.9	99±0.1	40±1.0	49±1.0
%2.5 PKP	9.88 ±0.03[ab]	0.53±0.05	10.82±0.29	….	….	….	….	….
% 5.0 PKP	9.24±0.01[c]	0.54±0.01	10.66±0.29	….	….	….	….	….
% 7.5 PKP	10.14±0.11[a]	0.55±0.02	10.38±0.28	….	….	….	….	….
% 10 PKP	10.02±0.13[ab]	0.56±0.04	10.34±0.28	….	….	….	….	….

[1]: Means marked with the same letter and without letters are not statistically different from each other (p>0.05); means given with different letters are statistically different from each other (p<0.05).
[2]: Ash content in dry matter.
[3]: Protein contents were calculated by multiplying the total nitrogen amounts by 5.7 for the control bread and 6.25 for the other samples.
[4]: Flour without palm kernel powder.

Table 2. Farinograph and Extensograph analysis parameters of control and flour blends[1].

PKP Ratio	Farinograph values				Extensograph values[2]			
	Water absorbtion[3] (%)	Development time (min.)	Stability (min.)	Softening degree[4] (BU)	Energy (cm^2)	Resistance to Extension (BU)	Extensibility (mm)	Maximum Resistance (BU)
Control[5]	60.75±0.62	1.8±0.00	7.65±0.65^{c}	58.5±6.36^{a}	100±7	765±47	107±7.5	841±7.5
%2.5 PKP	61.4±0.45	1.9±0.10	11.1±0.40^{b}	47.5±7.78^{ab}	93±0.50	777±54.5	99.5±9	855±9
% 5.0 PKP	61±0.55	1.95±0.25	12.7±0.40^{a}	41.0±1.41^{b}	89.5±8	800.5±84	94±8	864.5±8
% 7.5 PKP	61.15±0.62	2.0±0.20	13.45±0.15^{a}	38.5±3.53^{b}	89±6	810±66.5	93±10.5	879±10.5
% 10 PKP	61.15±0.67	2.05±0.15	13.5±0.10^{a}	37.5±7.77^{b}	89±3	816.5±1	90.5±8.8	885±4

[1]: Means marked with the same letter and without letters are not statistically different from each other (p>0.05); means given with different letters are statistically different from each other (p<0.05).
[2]: Extensograph values are the values measured after 135 minutes.
[3]: Based on 14% humidity.
[4]: BU: Brabender Unit and ICC/12 min. later.
[5]: Flour without palm kernel powder.

Table 3. Bread analysis results.

PKP Ratio		Control[2]	2.5%	5.0 %	7.5%	10.0%
Weight (g)		129±0.26[d]	130.41±0.30[c]	131.69±0.09[b]	132.75±0.14[a]	133.42±0.00[a]
Volume (cc)		411±1.0[e]	424.5±2.50[d]	436.5±1.5[c]	469±2.00[b]	485±5.00[a]
Specific volume (cc/g)		3.18±0.01[d]	3.25±0.02[cd]	3.31±0.01[c]	3.53±0.02[b]	3.63±0.03[a]
Crust	*L**	61.46±0.36[a]	54.15±0.14[b]	51.15±0.82[c]	48.01±0.76[d]	45.58±0.53[e]
	*a**	10.9±0.11[a]	9.65±0.20[ab]	9.23±0.10[ab]	8.21±0.25[b]	7.28±0.17[c]
	*b**	29.46±0.41[a]	26.97±0.17[ab]	26.53±0.29[ab]	24.79±0.01[b]	22.99±0.09[c]
Crumb	*L**	73.92±0.09[a]	66.6±0.19[b]	62.66±0.19[c]	58.99±0.25[d]	56.11±0.35[e]
	*a**	-0.88±0.10[e]	1.18±0.03[d]	2.54±0.05[c]	3.075±0.05[b]	4.45±0.12[a]
	*b**	14.11±0.36[a]	13.18±0.26[b]	12.08±0.06[c]	10.91±0.11[d]	10.29±0.12[d]
Hardness (g)	1st day	454.82±2.70[c]	560.88±17.37[b]	620.27±16.86[ab]	657.18±5.36[ab]	865.41±12.26[a]
	3rd day	641.72±2.05[e]	1015.48±45.78[d]	1196.16±27.93[c]	1363.65±30.82[b]	1666.66±4.82[a]

[1]: Means marked with the same letter and without letters are not statistically different from each other (p>0.05); means given with different letters are statistically different from each other (p<0.05).
[2]: Flour without palm kernel powder.

3.2. Bread Analysis Results

Some physical analysis results of bread samples obtained from control and flour blends are summarized in Table 3. When the results are examined, it is seen that the bread weight and volume increase as the PKP ratio increases. In another study in which different proportions of menenges flour were added to bread wheat flour, bread volume increased in parallel with the increase rate (Ünüvar, 2013). The increase in the specific volume value is also compatible with the bread volume value. Color is one of the most prominent features in physical analysis. The color characteristics of the raw material used are reflected in the final product. One of the most important factors affecting consumer choice is the visual feature of the product. In this study, it was determined that the brightness value *(L* value)* of both the crust and the inner color of the bread decreased in parallel with the increasing usage rate. On the other hand, *a* value* decreased in the crust part as the PKP ratio increased, but increased in the crumb (Table 3). The reason for this situation may be due to the reaction that develops because of the Maillard reaction that occurs on the surface of the bread during baking (Demir *et al*., 2006).

When the hardness measurements were carried out, it was determined that the hardness value increased due to the increased PKP ratio on the 1st and 3rd days (Table 3). The amount of fiber in the components that make up the bread affects the nutritional value of the bread as well as its hardness and staling mechanism. Dursun, (2016) used fish meat for the purpose of enriching the bread in his study and stated that the hardness of the bread increased as time passed (24-48-72 hours). The result obtained in our study is similar to this literature.

4. DISCUSSION and CONCLUSION

In this study, the effects of palm kernel powder (PKP) on dough rheology and bread properties were investigated. The results showed that the increase in PKP and some farinograph values showed a statistically significant difference ($p<0.05$), and as a result, the bread value was also positively affected. The purpose of additives made on the basis of the displacement principle in foods is to improve the quality of the final product and to increase the nutritional and functional value. Studies conducted for this purpose are aimed at determining the optimum ratios of the additives to be used. Since there is limited literature on the use of PKP as a functional food, the original value of this research is thought to be high. It is important to increase this and similar reviews in terms of making a positive contribution to the environment by evaluating the waste and improving the nutritional and technological properties of bread.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors

Authorship Contribution Statement

Yavuz Yuksel: Investigation, Resources, Visualization, Software, Analysis, Original draft. **Mustafa Kursat Demir**: Supervision, and Validation, Analysis, Reading and writing the manuscript, Editing.

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Research Article

Determination of secondary metabolite in galls of some cynipid wasps (Hymenoptera: Cynipidae) and characterization of the phenolic compound

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Abstract: The galls of cynipid species (Hymenoptera: Cynipidae) have been used since ancient times as an important source of bioactive compounds. Many researchers have evaluated the medicinal potential of some cynipid galls and found that these galls have numerous ethnomedical uses. The aim of this study was to determine the total bioactive (phenolic, flavonoid and tannin) compound amounts of gall extracts, to reveal the phenolic compound contents by HPLC method and to set ground for future pharmaceutical studies. The galls of cynipid wasps (totally 24 taxa) on host plants were collected from the Eastern Black Sea Region of Türkiye. Acetone, ethanol, methanol, and water extracts of these galls were prepared for quantity analysis and HPLC. The phenolic compound amounts (phenolic, flavonoid and tannin) of the cynipid gall extracts were determined, and their phenolic compound contents were also revealed. Some phenolic compounds in ethanol gall extracts were analyzed using HPLC, and some of these compounds were detected for the first time in the cynipid galls. 2,5-dihydroxybenzoic acid, caffeic acid, epicatechin, and ellagic acid are the most abundant in the ethanolic gall extracts. Total phenolic, flavonoid and tannin amounts of the cynipid gall extracts showed high variation. All these studies on quantification and characterization of phenolic compound are the first detailed studies on these taxa of cynipid galls and show that these cynipid galls might pharmaceutically be an important source for human and animal health.

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1. INTRODUCTION

Phenolic compounds, known more than 8.000 structures, are the most widely distributed secondary metabolites in plants (Del Rio *et al*., 2013; Mammadov, 2014; Vuolo *et al*., 2019). The accumulation of phenolic compounds, which play very important roles in metabolism, in the plant cell is important for the life of the plant (Mammadov, 2014). The production of the phenolic compound by plants enables them to cope with changing environmental challenges (intense light, low temperature, nutrient deficiency, etc.) throughout the course of evolution (Lattanzio, 2013). Phenolic compounds act as protective agents, inhibitors, natural toxic substances, and pesticides to defend natural plants against herbivores, nematodes, phytophagic

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insects and pathogens (fungi and bacteria) (Cornell & Hawkins, 2003; Bhattacharya *et al*., 2010; Lattanzio, 2013). In addition, they contribute to the nutritional, colour, and sensory properties of vegetables and fruits (Chikezie *et al*., 2015). Currently, numerous scientific literature reports considered as important compounds for human health owing to their antioxidant activity, antidiabetic, antimutagenic, anti-inflammatory, cardioprotective, neuroprotective, antitumor, and antiaging properties, etc. (Zhang *et al*., 2018; Vuolo *et al*., 2019).

Host plants defend their own tissues through secondary metabolite production and accumulation instead of staying silent against herbivorous insects (Fürstenberg-Hägg *et al*., 2013). Gall-inducing groups in insects affect plant tissues for their own benefit (Mani, 1964; Stone & Schönrogge, 2003; Giron *et al*., 2016; Oliveira *et al*., 2016). It is thought that the secondary metabolite accumulation induces gall development on host plant (Oliveira *et al*., 2014). The host plants form a new functional structure (gall) to protect their vascular bundles (Isaias *et al*., 2013). The gall provides a microenvironment in which insects can feed and develop (Price *et al*., 1986, 1987; Stone & Schönrogge, 2003). As a result, plants have found the best way in the evolutionary process by offering them food sources instead of chemical counterattack against gall-inducing insects (Stone & Schönrogge, 2003).

In accordance with the histochemical research, it was revealed that the most common secondary metabolite groups in galls are: (1) phenolic compounds, (2) terpenes and (3) alkaloids (Kuster *et al*., 2020). In the inner and outer tissues of galls, phenolic compounds are found in different proportions. Phenolic compounds, which are rare in inner tissues, allow insect larvae to feed on these tissues (Abrahamson *et al*., 1991; Bronner 1992; Isaias *et al*., 2000; Cuevas-Reyes *et al*., 2004; Detoni *et al*., 2010; Ferreira *et al*., 2017). While the metabolites accumulated in the outer tissues of the galls protect the gall-inducing insects, the inner tissues serve as a food source (Bragança *et al*., 2017). The species-specific morphological structure of galls depends on the storage of phenolic compounds in different gall tissues. The conservative feature of gall tissues due to their phytochemical content is seen in all galls, regardless of the herbivore taxa (Kuster *et al*., 2020).

Gall wasps or cynipids belonging to Cynipidae (Insecta: Hymenoptera) are one of the important insect groups that induce gall on host plants. The most important host plants of gall-inducer cynipids, which has roughly 1.400 species in the world (Buffington *et al*., 2020) and 165 species in Türkiye (Azmaz & Katılmış, 2017, 2020a, 2020b, 2021a, 2021b; Azmaz, 2021; Bayrak & Avcı, 2019; Mutun *et al*., 2020; Demirel *et al*., 2022, 2023; Fatih & Gençer, 2022; Tataroğlu & Katılmış, 2022), are oaks (*Quercus* L.), other Fagaceae genera closely related to oaks (*Castanea* Miller, *Castanopsis* (D.Don) Spach, *Chrysolepis* Hjelmq., *Lithocarpus* Blume, *Notholithocarpus* Manos, Cannon & S.H.Oh), and roses (*Rosa* L.). Besides, other plant families (such as Asteraceae, Lamiaceae, Rosaceae and Papaveraceae) are host plant groups for gall wasps (Ronquist *et al*., 2015; Buffington *et al*., 2020).

The cynipid galls have been used in folk medicine owing to their therapeutic properties since ancient times (Oefele, 1933; Schimitschek, 1953; Imtiyaz *et al*., 2013; Iminjan *et al*., 2014; Elham *et al*., 2021). Much research on cynipid gall extracts in the last two decades has revealed various biological activities of the cynipid galls (Gao *et al*., 2018; Iylia Arina & Harisun, 2019; Azmaz *et al*., 2020; Hu *et al*., 2020; Kılınçarslan Aksoy *et al*., 2020; Yusof & Abdullah, 2020). It is very important to determine the phytochemical (phenolic compound, flavonoid etc.) contents of the cynipid galls, which have high tannin content and different phenolic compounds (Taper & Case, 1987). Therefore, the study aimed to investigate the amounts of the total phenolic compounds (phenolic, flavonoid, and tannin) of the gall extracts belonging to different cynipid species. Besides, it was aimed to compare their amounts of phenolic compounds of all extracts, and to reveal characterization of the phenolic compounds for future studies as well.

2. MATERIAL and METHODS

2.1. Collection of Cynipid Galls and Preparation of Gall Extracts

Galls of cynipid species (Cynipidae) were collected from host plants (mostly oak species) distributed in the Eastern Black Sea Region, Türkiye between 2017 and 2019. In total, the galls of 20 different cynipid species were collected from their host plant to obtain the extract (Table 1). In addition, the galls of the four cynipid species (*Andricus assarehi*, *A. kollari*, *A. quercustozae*, and *Cynips quercusfolii*) were also collected from different host plants for comparison (Table 1). The cynipid species were identified by the Entomology Research Laboratory at Pamukkale University. Extracts and secondary metabolite studies were carried out in the Secondary Metabolite Laboratory, Pamukkale University.

Table 1. Cynipid galls collected and their host plants from the study area.

Cynipid Galls	Host Plant	Abbreviations of Gall Extracts in Tables 2-6
Asexual galls of *Andricus assarehi* Melika & Sadeghi, 2008	*Q. macranthera* subsp. *syspirensis*	AAQM
Asexual galls of *A. assarehi*	*Q. petraea* subsp. *iberica*	AAQP
Asexual galls of *A. caputmedusae* (Hartig, 1843)	*Q. macranthera* subsp. *syspirensis*	ACQM
Asexual galls of *A. fecundatrix* (Hartig, 1840)	*Q. petraea* subsp. *iberica*	AFQP
Asexual galls of *A. infectorius* (Hartig, 1843)	*Q. petraea* subsp. *iberica*	AIQP
Asexual galls of *A. kollari* (Hartig, 1843)	*Q. macranthera* subsp. *syspirensis*	AKQM
Asexual galls of *A. kollari*	*Q. petraea* subsp. *iberica*	AKQP
Asexual galls of *A. lignicolus* (Hartig, 1840)	*Q. petraea* subsp. *iberica*	ALQP
Asexual galls of *A. mitratus* (Mayr, 1870)	*Q. petraea* subsp. *iberica*	AMQP
Asexual galls of *A. polycerus* (Giraud, 1859)	*Q. macranthera* subsp. *syspirensis*	APQM
Asexual galls of *A. quercustozae* (Bosc, 1792)	*Q. infectoria*	AQQI
Asexual galls of *A. quercustozae*	*Q. macranthera* subsp. *syspirensis*	AQQM
Asexual galls of *Aphelonyx cerricola* (Giraud, 1859)	*Q. cerris*	ACQC
Asexual galls of *Ap. persica* Melika, Stone, Sadeghi & Pujade-Villar, 2004	*Q. cerris*	APQC
Sexual galls of *Biorhiza pallida* (Olivier, 1791)	*Q. petraea* subsp. *iberica*	BPQP
Asexual galls of *Cynips baskalei* Azmaz & Katılmış, 2020	*Q. petraea* subsp. *iberica*	CBQP
Asexual galls of *C. korsakovi* Belizin, 1961	*Q. macranthera* subsp. *syspirensis*	CKQM
Asexual galls of *C. quercus* (Fourcroy, 1785)	*Q. petraea* subsp. *iberica*	CQQP
Asexual galls of *C. quercusfolii* (Linnaeus, 1758)	*Q. macranthera* subsp. *syspirensis*	CQFQM
Asexual galls of *C. quercusfolii*	*Q. petraea* subsp. *iberica*	CQFQP
Sexual galls of *Diplolepis fructuum* (Rübsaamen, 1895)	*Rosa canina*	DFRC
Sexual galls of *D. mayri* (Schlechtendal, 1876)	*R. canina*	DMRC
Sexual galls of *D. rosae* (Linnaeus, 1758)	*R. canina*	DRRC
Sexual galls of *Synophrus politus* Hartig, 1843	*Q. cerris*	SPQC

Thirty-sixty galls without adults/larvae (depending on the gall size) belonging to each cynipid species were dried in the shadow and broken into small pieces with an electric blender. Samples of small gall part were transferred into beakers. Acetone, ethanol, methanol and water (dH_2O) were separately added in the ratio of 1:10 and were put in water bath at 55ºC for 6 h. The extraction mixture was separated from the residue using filter paper. This process was

repeated twice. Then, the solvents (acetone, methanol, and ethanol) of extract samples were removed using a rotary evaporator (BUCHI, rotavapor R-210/R-215, Germany). Also, the water extract was lyophilized using a freeze dryer (Thermosavant Modulyo D, USA). After removing the solvents, the gall extracts were obtained (Mammadov *et al*., 2011) and stored in the Entomology Research Laboratory, Pamukkale University, Türkiye.

2.2. Determination of Total Phenolic Components

2.2.1. *Quantification of total phenolic compound*

The Folin–Ciocalteu method (Slinkard & Singleton, 1977) with slight modification was used to determine the total phenolic amounts of each gall extract prepared with different solvents (acetone, ethanol, methanol, and water). The sample solution (1 mg/mL) was mixed with diluted Folin–Ciocalteu reagent (1 mL) and dH_2O (46 mL). After 3 min, sodium carbonate solution (3 mL, 2%, Na_2CO_3) was added. The absorbance of the mixture was measured at 760 nm after the incubation (in the dark, 2 h, room temperature). The total phenolic amount was expressed as equivalents of gallic acid (mgGAEs/g).

2.2.2. *Quantification of total flavonoid compound*

The total flavonoid amounts of each gall extract prepared with different solvents (acetone, ethanol, methanol and water) were analyzed according to the method by Arvouet-Grand *et al*. (1994). Aluminium trichloride (1 mL, 2% $AlCl_3$) was mixed with the same volume of extract solution (2 mg/mL). The absorbance was measured at 415 nm after the incubation (10 min, room temperature). The total flavonoid amount was expressed as equivalents of quercetin (mgQEs/g).

2.2.3. *Quantification of total tannin compound*

The vanillin method (Bekir *et al*., 2013) with slight modification was used for analyzing the total tannin amount of gall extracts prepared with different solvents (acetone, ethanol, methanol and water). The solution (0.5 mL) was mixed with vanillin reagent (1.5 mL, 1% in 7 M H_2SO_4) in an ice bath. The solution absorbance was measured at 500 nm after the incubation (15 min, room temperature). The total tannin amount was expressed as equivalents of (+)-catechin (mgCEs/g).

2.3. Characterization of Phenolic Compounds

The phenolic compound amounts of the ethanolic gall extracts were analyzed by high-performance liquid chromatography (HPLC) according to the method described by Caponio *et al*. (1999) with some modifications. Ethanol gall extracts were preferred for the HPLC method because ethanol dissolves only polar substances (like phenolic compounds) owing to it being a polar solvent. Detection and quantification were performed with a diode array detector (SPD–M20A), a pump (LC–20AT), a column heater (CTO–10ASVp), autosampler (SIL–20ACHT), the system controller (SCL–10Avp) and degasser (DGU–14A). The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, quercetin, and cinnamic acid were used as standards. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as µg per gram of extract.

2.4. Statistical Analysis

The SPSS Statistical Package program (SPSS Statistics Version 25) was used to analyze the results. The results were presented as mean ±SD. Since the data did not show normal distribution (Kolmogorov-Smirnov Test, $p>0.05$), differentiation among the extracted groups

was tested using the Kruskal-Wallis H Test, which is a non-parametric test used for many groups, was performed. Besides, it was statistically performed pairwise comparisons among groups. As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

3. RESULTS

The amounts of total phenolic, total flavonoid, and total tannin compounds of cynipid gall extracts were determined. When acetone extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest amount of total phenolic (378.73±13.6 mgGAEs/g), while the extract of the *A. polycerus* galls had the highest amount of total flavonoid (108.85±3.37 mgQEs/g) and the extract of *B. pallida* galls had the highest amount of total tannin (205.05±5.55 mgCEs/g). It was observed that there were significant differences among the groups in terms of the amounts of phenolic, flavonoid and tannin of acetone gall extracts (p<0.05). On the other hand, there were no statistically significant differences among the acetonic extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 2).

Table 2. Total amounts of phenolic, flavonoid and tannin compounds of acetone gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	378.73±13.6 ab	35.9±2.33	21.04±1.13 c
AAQP	148.10±9.81	29.90±0.50	90.68±14.84
ACQM	273.93±13.70	49.44±0.63	169.22±9.94
AFQP	184.77±4.01	59.41±1.27	14.61±0.03 ab
AIQP	281.64±7.63	59.19±0.25	25.73±1.18
AKQM	249.35±18.58	55.83±2.92	30.69±0.17
AKQP	284.56±10.29	64.29±0.84	192.83±2.88 a
ALQP	295.39±0.35	101.66±0.38 a	149.50±5.77
AMQP	87.89±9.38	9.90±0.27 ab	113.12±0.13
APQM	225.60±17.63	108.85±3.37 bc	113.20±0.00
AQQI	189.77±8.32	35.81±1.25	180.33±3.62
AQQM	103.31±10.84	29.10±0.41	30.68±4.17
ACQC	217.27±16.03	50.24±1.45	121.99±7.63
APQC	294.98±13.75	59.30±0.44	37.55±0.19
BPQP	239.98±8.48	50.44±0.67	205.05±5.55 bc
CBQP	216.43±14.07	43.73±0.02	97.55±6.47
CKQM	113.31±4.96	12.08±0.22	67.74±5.84
CQQP	73.93±5.55 b	24.01±1.43	82.83±5.83
CQFQM	82.06±4.88	11.77±0.17	121.16±3.81
CQFQP	167.68±11.97	11.48±0.13 c	84.11±2.82
DFRC	279.98±22.72	31.01±1.16	39.40±3.61
DMRC	271.44±17.50	25.16±0.25	35.81±2.00
DRRC	227.27±5.01	31.35±0.01	25.50±0.71
SPQC	1.43±0.62 a	33.34±1.12	83.24±0.95

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When ethanol extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest total phenolic compound (349.35±15.94 mgGAEs/g), while the extract of the *A. lignicolus* galls had the highest total flavonoid compound (102.01±0.32 mgQEs/g) and the extract of *A. quercustozae* galls (collected from *Q. infectoria*) had the highest total tannin compound (112.55±8.38 mgCEs/g). It was revealed that the amounts of phenolic, flavonoid and tannin compound of ethanol gall extracts were significantly different from each other (p<0.05). However, it was not found statistically significant differences among the ethanol extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 3).

Table 3. Total amounts of phenolic, flavonoid and tannin compounds of ethanol gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	349.35±15.94 cd	42.57±0.27	5.98±0.30
AAQP	165.60±8.48	40.60±0.19	21.07±0.84
ACQM	244.56±8.67	76.41±2.06	34.77±1.73
AFQP	192.27±9.39	63.84±0.11	8.52±0.15
AIQP	217.27±18.46	81.35±0.64 b	10.87±0.72
AKQM	237.47±11.61	54.31±0.49	1.82±0.47 b
AKQP	314.14±4.60 a	72.37±0.66	70.61±7.69
ALQP	288.93±10.32	102.01±0.32 cd	38.94±5.02
AMQP	128.73±5.08	19.39±0.11	36.28±0.80
APQM	207.69±9.01	83.06±4.81 a	12.60±0.27
AQQI	145.18±7.20	41.41±0.02	112.55±8.38 abc
AQQM	185.18±12.07	32.03±0.27	10.25±0.21
ACQC	155.81±13.91	61.88±0.41	76.16±2.20
APQC	314.56±12.07 b	67.91±0.82	3.18±0.08
BPQP	240.81±4.10	63.83±0.03	71.16±3.62
CBQP	148.72±8.86	60.56±0.53	74.22±5.54
CKQM	146.64±3.65	14.46±0.29	1.80±0.19 c
CQQP	52.48±3.44	10.76±0.27 d	66.16±5.83
CQFQM	30.81±2.50 d	11.09±0.08	15.33±1.44
CQFQP	73.94±2.50	9.08±0.24 abc	1.26±0.11 a
DFRC	286.22±11.27	23.87±0.28	20.22±0.42
DMRC	271.85±8.39	32.78±0.04	11.58±0.67
DRRC	261.22±0.72	44.47±0.23	9.66±0.23
SPQC	7.06±1.25 abc	16.58±0.13	14.90±0.81

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When methanol extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest total phenolic amount (403.73±1.57 mgGAEs/g), while the extract of the *A. lignicolus* galls had the highest total flavonoid amount (102.17±0.19 mgQEs/g) and the extract of *A. caputmedusae* galls had the highest total tannin amount (109.22±5.09 mgCEs/g). It was found that phenolic, flavonoid and tannin amounts of methanol gall extracts were significantly different from each other (p<0.05), while there were

no statistically significant differences among the methanol extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 4).

Table 4. Total amounts of phenolic, flavonoid and tannin compounds of methanol gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	403.73±1.57 bc	51.36±0.43	7.91±1.35
AAQP	255.81±11.57	52.84±1.73	15.31±0.87
ACQM	233.94±8.75	62.03±0.47	109.22±5.09 bc
AFQP	205.39±3.65	76.52±1.96	7.54±0.53
AIQP	227.27±17.69	97.27±0.90 a	9.73±0.29
AKQM	234.14±12.63	71.07±0.97	2.62±0.19
AKQP	264.98±7.91	73.82±0.74	63.94±4.11
ALQP	388.52±6.56 a	102.17±0.19 bc	43.66±2.88
AMQP	106.23±4.15	20.34±0.33	43.55±2.41
APQM	283.31±10.95	58.02±0.21	12.05±0.30
AQQI	200.81±7.80	42.87±0.67	78.94±6.36
AQQM	188.31±6.25	42.21±0.17	12.11±0.13
ACQC	113.94±1.25	88.46±0.41	78.66±8.03
APQC	343.93±9.20	78.99±1.22	2.77±0.20
BPQP	222.89±3.14	65.36±0.65	101.16±1.66 a
CBQP	187.48±19.59	71.83±1.68	67.83±4.63
CKQM	185.81±12.17	11.67±0.16 c	1.90±0.17 c
CQQP	75.39±3.76	10.58±0.39 ab	59.22±1.73
CQFQM	42.68±4.09 c	16.43±0.27	6.44±1.27
CQFQP	99.14±4.51	12.56±0.40	1.65±0.09 ab
DFRC	243.93±21.00	35.59±2.06	20.16±1.07
DMRC	277.89±3.14	50.80±1.14	14.87±0.31
DRRC	270.18±2.72	55.68±0.37	10.91±0.10
SPQC	22.89±1.90 ab	17.64±0.16	25.82±0.82

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When water extracts of the cynipid galls were compared, the extract of the *A. kollari* galls (collected from *Q. petraea*) had the highest total phenolic content (342.06±6.58 mgGAEs/g), while the extracts of the *A. assarehi*, *Ap. cerricola*, *B. pallida* and *C. baskalei* galls had the highest total flavonoid content and the extract of *Ap. cerricola* gall had the highest total tannin content (84.22±8.39 mgCEs/g). It was determined that phenolic, flavonoid and tannin contents of water gall extracts were significantly different among groups (p<0.05). On the other hand, there was not a statistically significant difference among the water extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 5).

Table 5. Total amounts of phenolic, flavonoid and tannin compounds of water gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	241.23±3.20	53.67±0.21	3.27±0.16
AAQP	262.89±5.63	118.42±0.00 a	5.71±0.68
ACQM	176.22±6.88	74.25±0.47	82.55±3.36 b
AFQP	205.60±0.95	97.45±0.96	5.32±0.10
AIQP	176.43±17.12	109.65±0.45	8.42±0.68
AKQM	221.23±7.55	73.85±1.07	2.12±0.19
AKQP	342.06±6.58 bc	100.39±2.65	80.05±4.28 a
ALQP	260.81±4.88	103.44±0.37	37.55±3.15
AMQP	186.85±5.24	117.35±1.85	8.95±0.64
APQM	187.27±7.50	96.47±2.96	7.46±0.28
AQQI	242.89±8.88	61.19±1.44	48.66±2.88
AQQM	178.10±3.14	58.75±0.87	7.13±0.39
ACQC	199.67±5.94	118.40±0.02	84.22±8.39 c
APQC	327.68±1.65 a	112.93±0.43	4.03±0.31
BPQP	162.89±3.20	118.40±0.02	71.16±3.81
CBQP	136.85±10.48	118.40±0.02	68.67±5.00
CKQM	242.27±3.76	20.00±0.56	2.65±0.19
CQQP	44.35±5.39	17.09±0.20	47.83±5.83
CQFQM	40.60±2.52 c	12.07±0.27 a	22.83±1.66
CQFQP	64.98±1.30	13.05±0.14	0.41±0.00 abc
DFRC	219.98±13.92	41.95±1.19	19.51±0.65
DMRC	231.22±13.82	52.47±1.33	15.01±1.30
DRRC	259.77±7.71	84.11±1.86	6.70±0.19
SPQC	34.56±0.62 ab	14.03±0.24	16.55±0.20

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences ($p<0.05$). It is stated that there is no significant difference in the groups without any letter ($p>0.05$).

The characterization of the phenolic compounds of ethanolic gall extracts were determined by HPLC method using 15 standard phenolic compounds. 2,5-dihydroxybenzoic acid, caffeic acid, epicatechin and ellagic acid were the most abundant compounds in the gall samples. Caffeic acid was detected in 15 of the samples, followed by epicatechin in eight samples and 2,5-dihydroxybenzoic acid in only one sample (Table 6). The amount of standard phenolic compound of gall samples is given in Table 6.

Table 6. Phenolic compound characterization of ethanol gall extracts.

Extracts of Cynipid Galls	Gallic Acid (µg/g)	3,4-dihydroxybenzoic Acid (µg/g)	4-hydroxybenzoic Acid (µg/g)	2,5-dihydroxybenzoic Acid (µg/g)	Chlorogenic Acid (µg/g)	Vanillic Acid (µg/g)	Epicatechin (µg/g)	Caffeic Acid (µg/g)	*p*-coumaric Acid (µg/g)	Ferulic Acid (µg/g)	Rutin (µg/g)	Ellagic Acid (µg/g)	Naringin (µg/g)	Quercetin (µg/g)	Cinnamic Acid (µg/g)
AAQM	146.1	321.6	43.7	2958.1	68.0	515.7	5951.9*	5514.6	0.03	7.4	< LOD	2154.8	34.5	42.6	21.9
AAQP	1021.1	270.3	634.0	3909.8	103.0	626.9	26612.4	39786.0*	< LOD	< LOD	< LOD	5026.2	245.8	< LOD	286.7
ACQM	193.2	309.1	680.2	4664.9	39.8	452.5	133082.8*	37228.8	28.8	145.0	10.6	1525.1	2182.9	409.0	0.1
AFQP	781.0	534.2	604.5	7329.1	489.8	524.0	13833.8	203835.8*	0.03	13.2	365.0	1281.1	0.8	58.0	138.0
AIQP	1371.7	1333.3	1495.8	6692.1	1678.5	412.0	3185.8	192029.6*	145.0	239.6	789.8	5092.1	1619.1	2967.0	213.6
AKQM	196.3	298.8	161.2	2773.3	196.2	501.2	4425.9	8026.5*	8.0	< LOD	< LOD	479.2	89.4	19.7	17.4
AKQP	656.9	756.8	757.3	11696.4	531.4	1150.9	123037.3*	12812.5	20.2	87.3	89.5	2010.3	53.1	7.4	22.8
ALQP	557.5	784.3	999.7	23380.3	764.4	1064.2	129063.5*	48674.2	77.2	280.5	4.6	4885.5	6573.5	295.4	804.5
AMQP	0.1	2.2	1.4	36.1	0.1	7.3	62.8*	55.3	0.05	< LOD	< LOD	36.4	4.8	< LOD	0.7
APQM	1539.0	261.9	990.2	14137.6	855.7	2639.3	18985.5	74183.1*	< LOD	< LOD	< LOD	11704.1	154.9	1041.7	644.9
AQQI	277.3	291.7	540.9	2839.0	63.4	238.7	36106.6*	33252.7	31.5	132.3	874.9	1807.9	814.7	27.6	11.3
AQQM	958.0	106.9	10.4	3091.9	100.1	479.2	5284.1	5290.0*	9.5	42.1	< LOD	1130.2	249.5	110.1	5.0
ACQC	1645.5	222.8	498.7	5356.2	1006.5	457.9	740.7	7354.5*	12.0	13.3	190.9	713.5	0.7	324.8	86.7
APQC	1458.0	2235.0	699.3	31286.9*	1592.5	597.6	3382.8	29320.6	29.1	60.8	770.5	4410.8	20.9	214.5	262.5
BPQP	531.3	678.1	587.5	2838.9	33.8	256.5	27879.8*	12955.1	66.4	529.2	273.6	1536.2	1646.9	378.8	224.1
CBQP	1610.5	228.7	1006.5	5334.8	786.3	992.1	3501.1	115369.9*	3.3	< LOD	105.4	2161.8	0.7	554.9	15.4
CKQM	1455.8	426.8	263.5	8058.9	3859.4	513.7	19662.6	90831.9*	< LOD	< LOD	< LOD	1439.3	9069.7	< LOD	2.0
CQQP	585.6	189.5	195.6	5447.3	267.2	1023.9	20441.0	70714.4*	43.9	149.3	716.5	444.3	5023.6	3.2	13.0
CQFQM	446.6	192.9	206.6	3257.7	77.4	598.9	29164.7*	7757.2	22.2	4.2	234.5	288.2	2736.6	0.2	5.6
CQFQP	28.7	97.0	629.1	8614.3	386.2	690.5	26219.8	83604.7*	< LOD	< LOD	< LOD	2217.1	150.1	17.4	62.3
DFRC	32.6	298.0	454.5	7821.2	258.8	2979.0	2408.5	10828.2*	21.8	17.5	< LOD	2994.3	6.0	12.0	13.2
DMRC	289.6	2949.8	6732.5	82015.2	2636.4	13172.9	402573.4	701264.4*	961.1	383.3	43.1	53626.2	724.1	2.1	748.6
DRRC	1004.3	1611.1	1519.1	44437.5	1261.0	1057.8	866.4	68135.7*	0.3	70.8	427.9	8926.7	3270.9	412.5	278.9
SPQC	128.3	41.6	37.6	988.6	31.8	60.2	2039.9	3383.8*	< LOD	< LOD	< LOD	369.2	0.9	3.8	26.2
Retention Time (min)	6.8	10.7	15.7	17.2	18.2	19.2	21.3	22.7	26.1	30.1	45.6	47.7	49.7	70.4	71.1

LOD: Limit of Detection; * Maximum Value

4. DISCUSSION and CONCLUSION

In the past, the cynipid galls have been used in both Western and Eastern folks in traditional medicine against various diseases and have taken their place in codex books (The British Pharmaceutical Codex, etc.) (Galla, 1911; Larew, 1987; Yılmaz Sarıözlü & Kıvanç, 2011). In ancient Chinese medical sources, the cynipid galls have been used against many diseases such as cancer (Gao *et al*., 2018). Many studies have been carried out about the bioactive compounds and biological properties of the galls in especially oriental countries (Asif *et al*., 2012; Noori *et al*., 2015; Iylia Arina & Harisun, 2019; Kot *et al*., 2019; Azmaz *et al*., 2020; Sukor *et al*., 2020). The cynipid galls are known to have both a greater variety and greater amounts of bioactive compounds compared to their host plant (Hartley, 1998; Gao *et al*., 2018). However, although most of the phytochemical studies on galls today are about the identification or isolation of tannin compounds, the subject of future studies should be on other groups of secondary metabolites (flavonoids, terpenoids, etc.) contained in the galls.

Experiments carried out to reveal the amounts of phenolic compounds (phenolic, flavonoid and tannin) of different cynipid gall samples showed that gall samples prepared using different solvents contained large amounts of phenolic compounds and were different from each other in terms of their amounts of compound.

The gall samples with the high amount of phenolic, flavonoid, and tannin mostly belong to the same genus (*Andricus*). The amounts of phenolic compounds of galls developed both in oak buds or acorns (mostly *Andricus* spp. galls) and galls formed in *Rosa* fruits were found to be higher than galls formed in oak leaves. It has been determined that *S. politus* gall contains very low levels of phenolic compounds. The amounts of phenolic compounds (phenolic, flavonoid, tannin) contained in galls are related to where the galls develop on the host plant. In young plant tissues, secondary metabolites are synthesized in higher amounts than older plant parts due to their diverse metabolic activities (Achakzai *et al*., 2009; Barton & Koricheva, 2010; Chomel *et al*., 2016; Hussein & El-Anssary, 2019; Kariñho-Betancourt *et al*., 2019). Numerous studies have shown the effect of galling organisms on the host plant metabolism, either by inhibiting, maintaining or inducing the synthesis of new compounds (Rokas *et al*., 2003; Stone & Schönrogge, 2003).

The amount of phenolic compound in cynipid gall extracts (*A. assarehi, A. lignicolus, Ap. persica*) was found to be high. It is well established that secondary metabolites amount and types are affected by soil type (Eyüpoğlu, 1999; Özyazıcı *et al*., 2013; Mammadov, 2014). We consider that the differences in the amounts of phenolic compounds of gall extracts may be due to the soil type where the plant of gall is grown. In addition, the amount of phenolic compounds in galls is related to the part of the plant where the galls develop and also depends on host plant species. As a result, due to some factors such as these, differences in phenolic amounts of gall extracts were detected.

Historically, the cynipid galls have been used as a natural therapeutic resource in traditional medicine with more ethnopharmacological applications than modern medicine. In this study, cynipid galls belonging to different cynipid species were different and had the amounts of high phenolic compounds (phenolic, flavonoid, tannin) and because of this reason, they may be useful in pharmaceutical applications against various diseases, however further studies are required to test this hypothesis.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Musa Tataroglu: Investigation, Resources, Visualization, Methodology, Software, Formal Analysis, and Writing - original draft. **Ozge Kilincarslan Aksoy:** Investigation, Resources, Visualization, Methodology, Software, Formal Analysis, and Writing - original draft. **Yusuf Katilmis:** Resources, Methodology, Supervision, Writing-Reviewing, and Validation. **Ramazan Mammadov:** Resources, Methodology, Supervision, Writing-Reviewing, and Validation.

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Research Article

Comparative chromatographic analysis of phenolic compounds of *Liquidambar orientalis* plant cultivated under *in vitro* salt stress

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Abstract: In this study, the effects of boron salt stress on in vitro cultivated *Liquidambar orientalis* (*L. orientalis*), a relict-endemic plant species, and the resulting changes in its phenolic appearance were investigated. Salt stress can cause negative impact on plant growth and production, especially in species with low salinity and drought tolerance, affecting metabolite expression and somaclonal execution. To evaluate the effects of different boron salts on meristem regeneration and progression, clonal in vitro *L. orientalis* meristems were exposed to boric acid, sodium perborate, sodium metaborate, and disodium octaborate salts. When compared with the control group examples where salt application was not performed, the highest regeneration percentage was determined to be 100% with the application of 1 mg/L disodium octaborate. In terms of the shoot formation capacity index, it was determined to be 5 mg/L. With a value of 4.94, the application of sodium perborate yielded the best result. In *L. orientalis* plants, the greatest change in phenolic compounds due to boron salt applications was observed in the concentration of Quercetin with the sodium perborate salt application at 1 mg/L concentration.

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1. INTRODUCTION

Liquidambar orientalis Miller, called Amber-i Sail in ancient times, is a member of the Altingiaceae family (Figure 1a). The plant, distributed in the southwestern regions of Türkiye and also known as Anatolian sweetgum tree, is a relict endemic species with medicinal-aromatic properties (Alan *et al*., 2018). Anatolian sweetgum, which was included in the noble hardwood group by EUFORGEN (European Forest Genetic Resources Program) in 2001 and is accepted as a species that needs to be protected throughout Europe, is also included in the "Highly Threatened in the Medium-Term Future in Nature" category in the IUCN danger categories list (Ekim *et al*., 2000). The natural distribution area of individuals belonging to the population is only in Fethiye, Marmaris and Köyceğiz districts. However, the natural forests of this species

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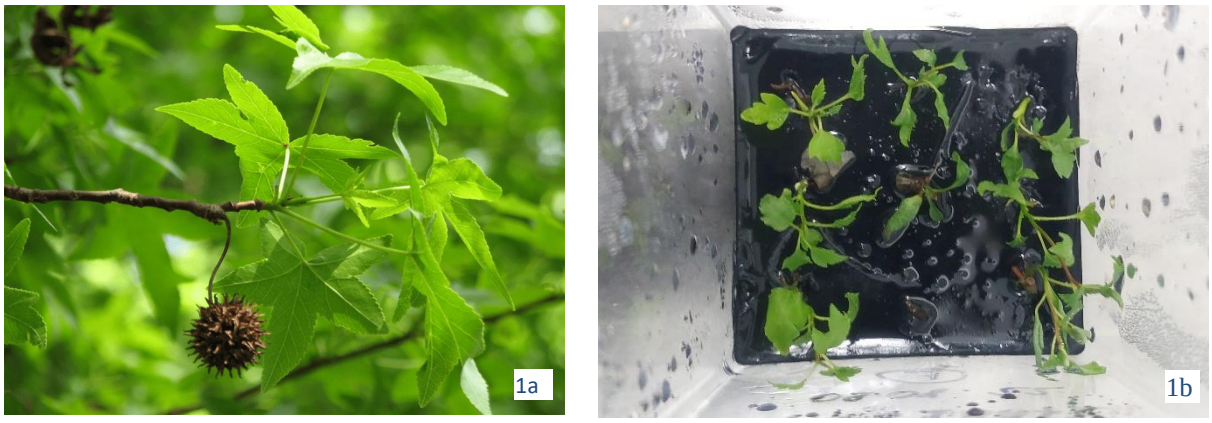
are in danger of extinction due to some reasons such as changing climatic conditions and destruction to create agricultural areas (Alan & Kaya, 2003). The oil with medicinal-aromatic properties obtained from the resin channels of the Anatolian sweetgum tree is an important raw material source for the cosmetics, pharmaceutical and chemical industries, which has been used and traded for thousands of years (Acar, 1989; Bozkurt *et al*., 1989).

Figure 1. *L. orientalis* plant in the natural population area (1a); clonal propagated seedlings *in vitro* (1b).



In vitro propagation techniques are the processes of producing more than one plant with the same genetic characteristics as that plant from organs such as shoots, roots, and stems, which can form a new *in vitro* micro-shoot (Figure 1b). It requires less material and is a faster production method than seed or steel production method (Ozudogru *et al.*, 2011; Kaya *et al.*, 2021). This technique, which is also used in the commercial sector, is used in many plant species in park-garden plants, agriculture, and forestry (Kaya *et al.*, 2018). Sometimes seed germination can take up to two years. Regeneration percentages are generally low. In addition, seed planting should be done seasonally. For these reasons, mass production is preferred commercially. Obtaining propagation material from tall trees is very difficult, thus it limits the success of clonal propagation using semi-mature shoot cuttings (Ozudogru *et al.*, 2013). Micropropagation offers an alternative method for producing Anatolian sweetgum for both commercial and conservation purposes (Bayraktar *et al*., 2015).

The use of *in vitro* techniques can reinforce *ex situ* and *in situ* conservation techniques for preserving plant genetic resources (Kaya *et al*, 2013). Axillary and/or apical shoot meristem-based *in vitro* propagation is one of the useful technologies used in plant biodiversity conservation (Souza *et al.*, 2017). Although boron salts and other salinity stresses and their effects on seed germination, shoot growth and development have been investigated in the literature (Munns & Termaat, 1986; Nable et al., 1997), there is still some uncertainty about the response of plants to combined stress caused by boron salts and other substances. Salinity stresses are usually found in waterlogged soils with high concentrations of boron and other salts in grains (Nable *et al*., 1997). In addition, naturally occurring high salinity can cause salinity stress in arid and semi-arid areas. In the current literature, there are limited studies on the effects of boron toxicity and salt stress on plant growth and development, and the response of plants to these stresses. There is no consensus on the interrelationships between boron toxicity and salinity stress (Keren & Bingham, 1958).

Boric acid is a crucial micronutrient for plants, influences various fundamental processes such as cell wall formation, flowering, fruit development, and nutrient transport, while also it regulates nitrogen metabolism and enhancing stress tolerance, highlighting the vital role of its proper use in fertilizers for plant health and productivity (Mercan *et al.*, 2022).

Sodium perborate is a chemical compound, which is widely employed across diverse applications, ranging from laundry detergents to teeth whitening products. Renowned for its bleaching and stain-removing capabilities, it finds utility in cleaning agents (Mercan *et al.*, 2022). Sodium metaborate is a versatile chemical compound, which is extensively employed as a buffering agent, pH regulator, and corrosion inhibitor in industrial processes. Additionally, it is utilized in the formulation of cleaning products, detergents, and serves as a boron fertilizer in agriculture, thus these underscore its significance as a valuable compound offering diverse contributions to various industries. Disodium octaborate is a chemically significant compound with diverse applications; it is utilized for wood preservation in various industrial sectors, exhibits antifungal properties, and has been investigated under *in vitro* conditions in various plant species (Mercan *et al.*, 2022).

In this study, the aim of this study is to examine the effects of growing the *Liquidambar orientalis* plant under boron salt stress *in vitro* on the plant and the changes in its phenolic compounds. Phenolic compounds are important components due to their remarkable properties in terms of consumption of the plant and their important health effects as natural antioxidants in human life. In addition, phenolic compounds can also be used as natural sweeteners. Due to the carcinogenic and toxic effects of artificial antioxidants in terms of health, phenolic compounds as natural antioxidants can cause cataracts, senile diseases, cancer, etc. It is said to be good for many diseases. For these reasons, consuming products with a high amount of phenolic substances can reduce the risk of catching diseases and positively affect health.

2. MATERIAL and METHODS

2.1. Preparation of Woody Plant Medium (WPM)

2.462 g/L WPM ready-made nutrient medium and 30 g/L Sucrose were weighed and transferred to a beaker. Sterile distilled water was added to the beaker and mixed homogeneously with a magnetic stirrer. The pH was adjusted to 5.8 and transferred to an autoclave bottle containing 7g/L agar. Autoclaving was performed at 121.6 °C for 15 minutes, and then 1 L of WPM medium was transferred in a laminar flow cabinet with 50 mL of medium in each culture dish. After about 30 minutes, the solidified media were stored in a cooler at +4 °C

2.2 Seed Surface Sterilization and Planting

The seeds of sweetgum were washed under running water for 20 minutes and kept in distilled water at 23 °C for 2 hours, and the floating seeds on the water were removed. Seeds that settled to the bottom in water were used for the sterilization process. Seeds were shaken in falcon (50 mL) for 10 minutes in 70% Ethanol (C_2H_5OH), 10 minutes in 10% Hydrogen Peroxide (H_2O_2), and 10 minutes in 10% commercial Sodium Hypochlorite (NaClO) (Domestos®), respectively (Kaya *et al*, 2017; 2020). After each step, the seeds were rinsed with sterile distilled water and transferred to blotting paper. The dried seeds were transferred to the nutrient media in a laminar flow cabinet. Sowing was carried out in 20 culture vessels with 5 seeds in each medium. The sown culture pots were left to germinate in the climate chamber at 23 °C. The sweetgum seedlings that developed during the germination process were micro propagated 3 times at 6-8 weeks intervals (Mercan *et al.*, 2022). All tools and equipment used in the experimental stages were autoclaved at 121.6 °C for 15 minutes in an autoclave device (Daihan Scientific-MaXterile60).

2.3 Boron Salt Application

In the experiment, four different boron salts, disodium octaborate ($Na_2B_8O_{13}.4H_2O$), sodium metaborate ($NaBO_2$), boric acid (H_3BO_3) and sodium perborate ($NaBO_3.nH_2O$) compounds were prepared at three different concentrations (1, 3 and 5 mg/L) and 1 mg/L benzyl was combined in adenine (BA) based WPM medium. The media adjusted to pH 5.8 were

autoclaved. The meristems extracted *in vitro* conditions were transferred to the prepared media and 10 meristems were planted on each medium. The parameters were repeated 3 times. Culture dishes were incubated under standard culture conditions (16/8 h photoperiod, 25±2 °C, 50 μmol^{-1} m^{-2} sec^{-1}, white cold fluorescent light) for 4 weeks. Each salt treatment was evaluated separately. Within the growth medium, plant specimens were subjected to a triple replication, with ten plant samples utilized for each experimental condition. The vitality status of these plant samples was examined, and the average vitality ratio was calculated, followed by the determination of the regeneration rate. To evaluate shoot counts, the post-growth shoot numbers of the plant samples were carefully analysed, and the mean of the three control groups was computed for comparative analysis (Mercan *et al.*, 2022).

2.4 Identification of Phenolic Compounds by UPLC-MS/MS

Approximately 3g of each plant sample was taken and they were decomposed with 200mL of liquid nitrogen. A mixture of 30mL of acetone: water (80:20) was added and left to extract at -86 °C for 6 hours. After the mixture removed from the cooler was kept in an ultrasonic bath for 15 minutes, the extract was centrifuged at 4000rpm at 20°C for 10 minutes. It was filtered through Whatman No 4 filter paper and then the residue was extracted 2 more times with 30 mL of acetone: water mixtures. The acetone in the combined extracts was evaporated under low vacuum at 40°C (Rotary Evaporator Heidolph Basis Hei-VAP ML). The aqueous phase was washed 3 times with 30mL of n-hexane followed by diethyl ether followed by liquid-liquid extraction 3 times with 30mL of ethyl acetate. The organic phases were combined. It was dried by evaporation at 40°C and redissolved in water: methanol (80:20) mixture. The solution was passed through Macherey-Nagel Chromafil Xtra PTFE-20/25 0.20µm filters and analyzed by UPLC-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS-MS) (Kıvrak & Kıvrak, 2017).

3. RESULTS and DISCUSSION

3.1. Effects of Boron Salts on Meristem Regeneration and Shoot Growth

When the samples incubated in WPM medium containing four different boron salts at different concentrations were compared with the control group, the best results were obtained from 5 mg/L sodium perborate (Figure 2). However, meristems cut from *L. orientalis* micro shoots (Figure 3a) gave the best regeneration percentage (Figure 3b, Figure 4) in the medium containing 1 mg/L disodium octaborate, while maximum shoot numbers per meristem were obtained without application 3 mg/L boric acid (Figure 5).

The shoot-forming capacity of *L. orientalis* plants grown with 4 different boron salts was measured. As a result of the measurements, the control group showed a shoot count of 4, while plants grown with boric acid application had shoot counts sequentially from low to high concentrations as (4, 5, 3 mg/L). Plants grown with sodium perborate application had shoot counts sequentially from low to high concentrations as (4, 4, 5mg/L). For plants treated with sodium metaborate, shoot counts were sequentially from low to high concentrations as (4, 4, 2 mg/L). Finally, for plants treated with disodium octaborate, shoot counts were sequentially from low to high concentrations as (3, 3, 2 mg/L) (Figure 2).

Figure 2. The shoot-forming capacities index calculated after salt application (Mercan *et al.*, 2022).

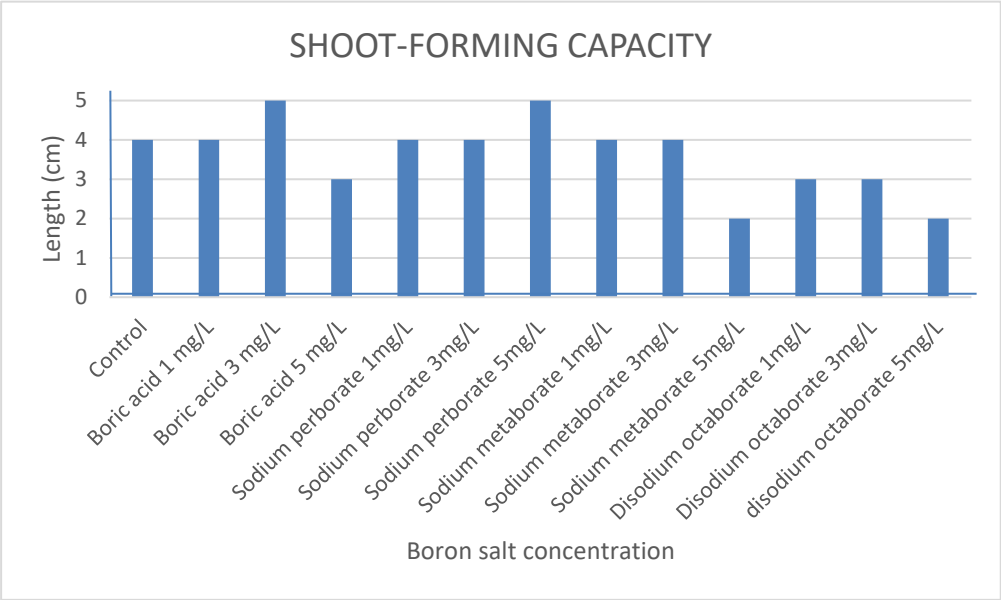
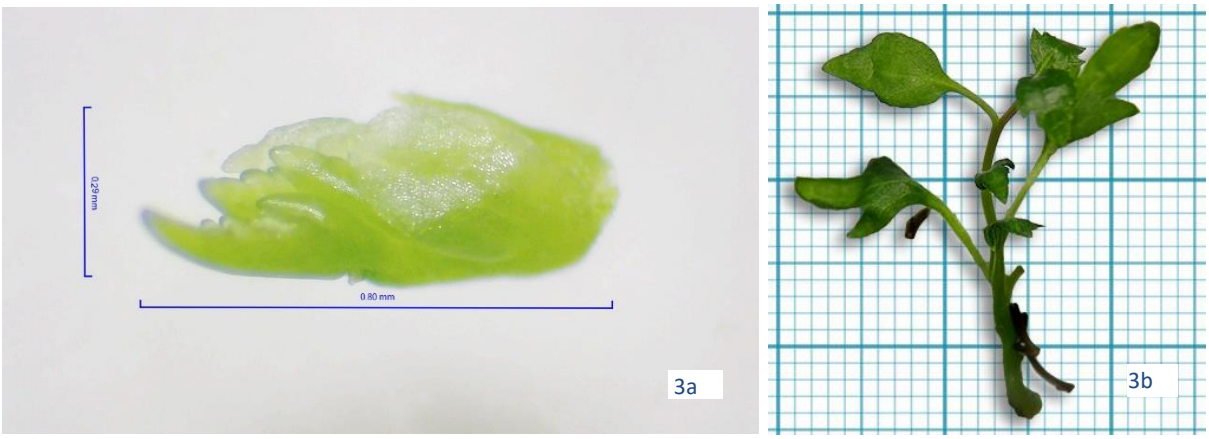
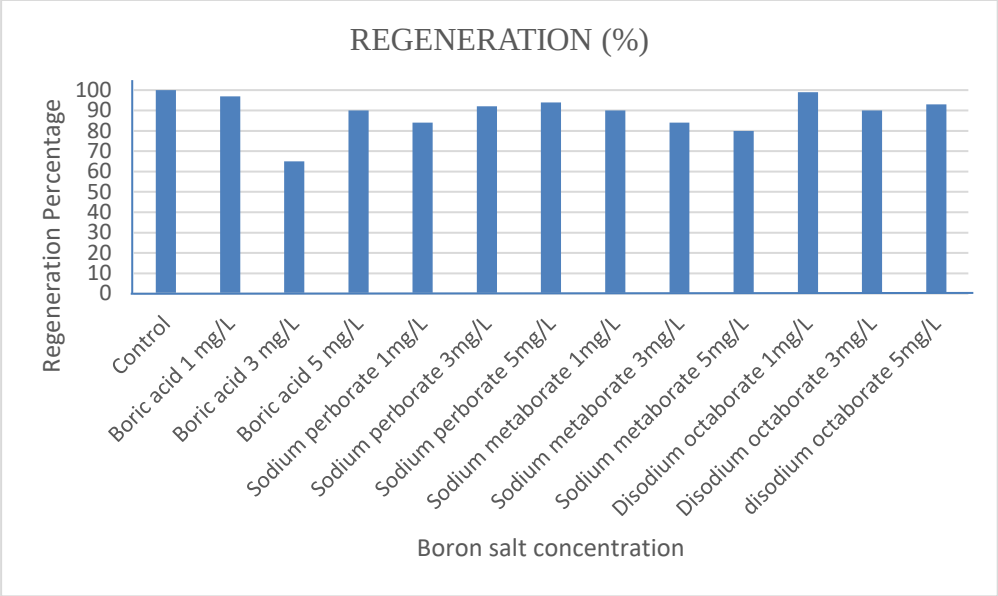


Figure 3. Meristem (3a) cut from *L. orientalis* micro-shoots, *L.orientalis* plant grown in WPM medium containing 1mg/L Disodium octaborate (3b) (Mercan *et al.*, 2022).



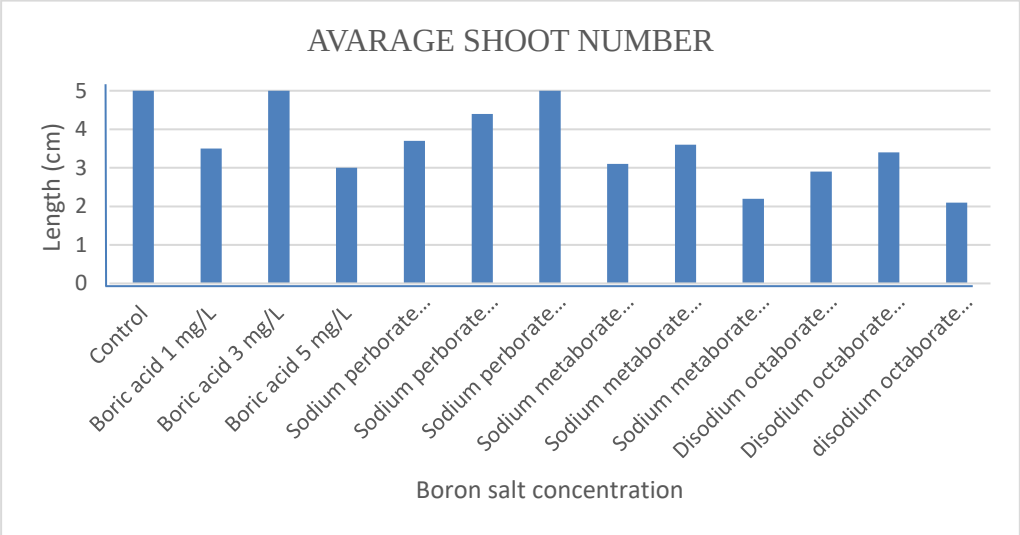
As a result of the cultivation of the control group, which was grown with 4 different boron salt stress and no salt application, the regeneration percentages were 100% in the control group. Regeneration percentages of plants grown with boric acid were measured from low concentration to high concentration (95%, 64%, 90%), respectively. The regeneration percentages of the plants grown with sodium perborate were measured from low concentration to high concentration (85%, 92%, 94%), respectively. Regeneration percentages of plants grown with sodium metaborate were measured from low concentration to high concentration (90%, 85%, 80%), respectively. The regeneration percentages of plants grown with disodium octaborate were measured from low concentration to high concentration (99%, 90%, 94%), respectively (Figure 4).

Figure 4. Meristem regeneration rates after different boron salt application (Mercan *et al.*, 2022).



The average numbers of meristems were counted for plants grown with 4 different boron salts at various concentrations. In the control group, the average meristem count for the grown plants was observed to be 5. However, for plants treated with boric acid salt, the average meristem counts were sequentially as (3.4, 5, 3cm) from low to high concentrations. Similarly, for plants treated with sodium perborate salt, the average meristem counts were sequentially as (3.7, 4.4, 5cm) from low to high concentrations. For plants treated with sodium metaborate salt, the average meristem counts were sequentially as (3.1, 3.6, 2.2cm) from low to high concentrations. Lastly, for plants treated with disodium octaborate salt, the average meristem counts were sequentially as (2.9, 3.4, 2.1cm) from low to high concentrations (Figure 5).

Figure 5. Meristem shoot number after different boron salt application (Mercan *et al.*, 2022).



In Table 1, the phenolic compound concentrations of the plants grown with 4 different boron salts and the control group, which were not subjected to salt stress, are given. As a result of the application of each salt at 3 different concentrations, the concentrations of plant phenolic compounds were measured in µg/kg. In Figure 6 and Figure 7, MRM chromatograms of quercetin and catechin hydrate phenolic compounds of major phenolic compounds of *L. orientalis* plant grown with 4 different boron salts applied at the same concentration are given.

Table 1. The effect of boron salts applied at different concentrations on the phenolic compounds (µg/kg) of the plant *Liquidambar orientalis*.

Phenolic compounds	Control	Boric acid			Sodium perborate			Sodium metaborate			Disodium octaborate		
		BA-1	BA-3	BA-5	SP- 1	SP- 3	SP- 5	SM- 1	SM- 3	SM-5	DO-1	DO-3	DO-5
Pyrogallol	6464.1	4479.6	4322.4	3986.5	6606.9	6444.5	5365.3	6865.1	5862.7	5273.9	6426.0	4781.7	5272.6
Gallic acid	4632.8	3541.6	3695.8	2961.2	5121.6	4760.6	4044.7	5034.4	4276.8	3878.4	4008.9	3191.4	3585.7
3-4-Dihydroxy benzoic acid	614.9	570.7	378.5	448.2	633.3	731.1	900.3	709.3	798.8	1022.1	686.5	653.9	516.3
Gentisic acid	900.8	872.2	607.4	746.0	915.2	1111.7	1294.7	1120.9	1111.2	1519.8	1025.2	1131.1	832.4
4-Hydroxy benzoic acid	315.9	408.1	194.4	449.9	198.1	278.6	273.0	172.8	207.6	320.4	287.3	387.9	322.6
Catechin hydrate	13742.1	16557.6	9383.5	5790.8	21813.6	17654.5	15312.0	15825.5	10008.4	13166.5	17653.7	20633.5	9424.1
Caffeic acid	574.1	330.5	438.9	542.7	375.6	513.8	624.3	482.8	529.9	568.5	272.5	387.5	407.3
p-Coumaric acid	1879.0	1284.1	467.1	1429.2	458.2	1078.8	894.9	1102.5	848.7	1837.8	533.2	981.2	497.1
Catechin gallate	2458.5	1077.2	1738.4	2376.7	471.6	568.1	537.1	739.9	790.7	901.8	1032.0	1069.5	704.0
Myricetin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Naringenin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Kaempferol	17.7	22.6	25.6	17.3	10.0	21.0	13.9	10.1	12.7	22.5	11.5	30.8	25.1
Epicatechin	26670.6	31374.1	12604.6	9878.7	44766.0	34950.0	27439.0	34081.8	24141.7	24798.7	34675.8	43448.1	17046.2
3,4-Dihydroxybenzaldehyde	131.2	146.5	103.3	120.9	172.0	171.4	145.9	178.5	161.5	152.6	144.3	135.9	132.8
Quercetin	12177.8	38407.6	9654.9	11367.8	42193.3	40304.6	45732.4	11197.5	21135.2	27701.1	16967.4	9447.6	6537.1

Data represent four independent experiments.
LOQ: Limit of Quantification
ND: Not detected

BA-1: 1 mg/L boric acid; BA-3: 3 mg/L boric acid; BA-5: 5 mg/L boric acid; SP-1: 1 mg/L sodium perborate; SP-3: 3 mg/L sodium perborate in; SP-5: 5 mg/L sodium perborate; SM-1: 1 mg/L sodium metaborate; SM-3: 3 mg/L sodium metaborate; SM-5: 5 mg/L sodium metaborate; DO-1: 1 mg/L disodium octaborate; DO-3: 3 mg/L disodium octaborate; DO-5: 5 mg/L disodium octaborate; Control: Plants without boron salt stress.

Figure 6. MRM chromatograms of Catechin hydrate phenolic in *L. orientalis* plant in which four different boron salts were applied at 3 mg/L concentration in UPLC-MS/MS device.

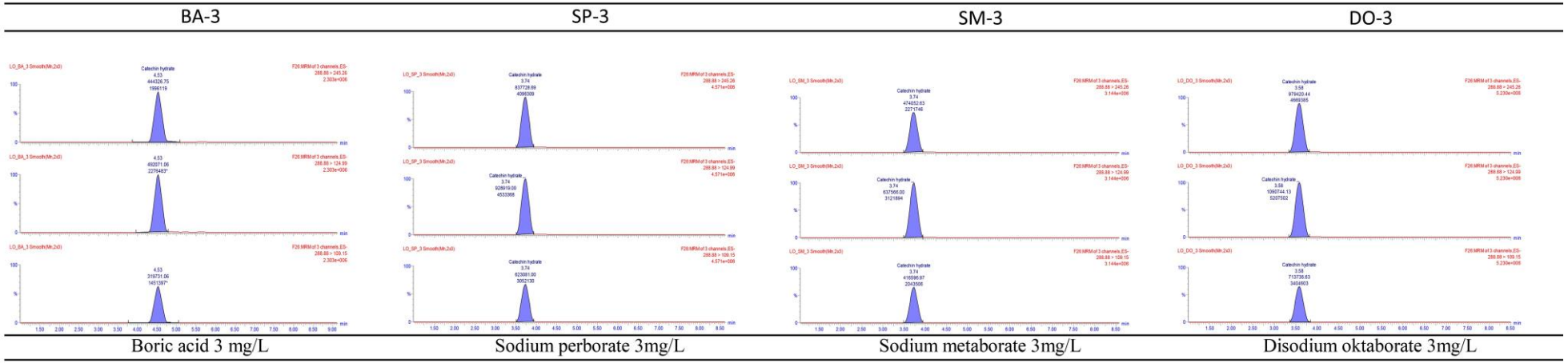
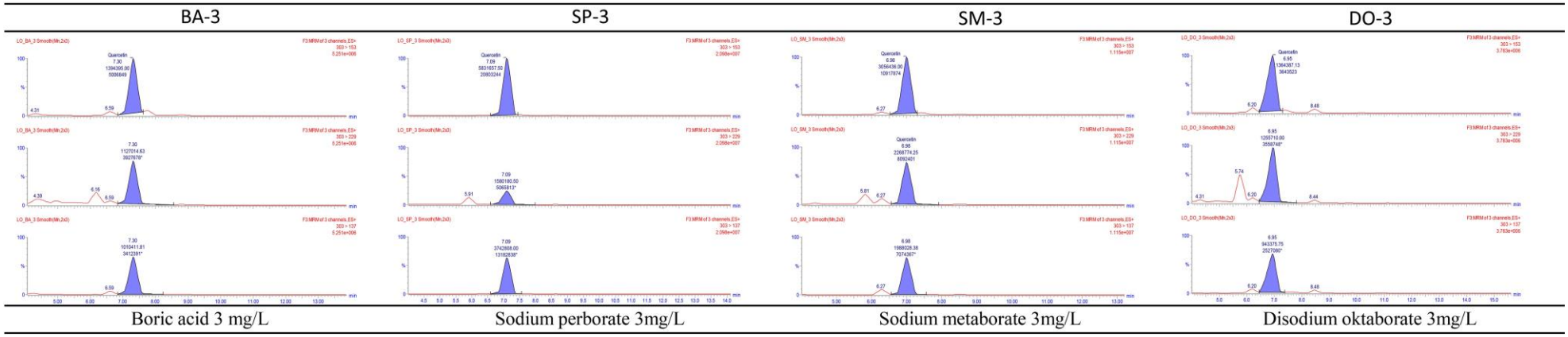


Figure 7. MRM chromatograms of Quarcetin phenolic in *L. orientalis* plant in which four different boron salts were applied at 3 mg/L concentration in UPLC-MS/MS device.



4. DISCUSSION and CONCLUSION

4.1. Effects of Boron Salts on Meristem Regeneration and Shoot Growth

In plants treated with boric acid salt, the average meristem counts were observed as 3, 4, 5, and 3 cm for low, medium, and high concentrations, respectively. These results indicate that at low concentrations, boric acid could potentially reduce the number of meristems in plants, but at medium concentrations, results similar to the control group were obtained. For plants treated with sodium perborate salt, the average meristem counts were observed as 3.7, 4.4, and 5cm for low, medium, and high concentrations, respectively. These findings suggest that sodium perborate may have the ability to enhance the meristem count in plants, particularly showing a significant increase at high concentrations. In plants treated with sodium metaborate salt, the average meristem counts were observed as 3.1, 3.6, and 2.2 cm for low, medium, and high concentrations, respectively. These results demonstrate that sodium metaborate can potentially decrease the meristem count at low and medium concentrations, with a more pronounced reduction observed at high concentrations. For plants treated with disodium octaborate salt, the average meristem counts were observed as 2.9, 3.4, and 2.1cm for low, medium, and high concentrations, respectively. These outcomes indicate that disodium octaborate might lead to a decrease in meristem count at both low and high concentrations, while results similar to the control group were achieved at medium concentrations (Figure 2).

Upon scrutinizing the effect of boric acid salt on regeneration percentages, it is discerned that at low concentrations, the regenerative abilities of plants exhibit a slight decrement. However, at moderate concentrations, regeneration percentages akin to those of the control group are attained. These outcomes signify that boric acid may endorse the regenerative potential of plants at suitable concentrations. Upon investigating the influence of sodium perborate salt, it is evident that at low concentrations, there is a marginal reduction in regeneration percentages, yet a conspicuous augmentation is observed at both intermediate and high concentrations. These findings postulate that sodium perborate has the potential to amplify the regenerative capacities of plants, particularly under conditions of heightened stress. Analysis of the impact of sodium metaborate salt reveals that at low and moderate concentrations, there is a decline in regeneration percentages, while a more pronounced reduction is evident at elevated concentrations. These outcomes suggest that heightened levels of sodium metaborate could detrimentally affect the regenerative capabilities of plants. Exploring the effect of disodium octaborate salt, it is ascertained that reductions in regeneration percentages manifest at both low and high concentrations, although outcomes closely approximate those of the control group at intermediate concentrations. These results intimate that disodium octaborate has the potential to bolster plant regeneration at appropriate concentrations (Figure 4).

When scrutinizing the effect of boric acid application on shoot counts, it is evident that at low concentrations, there is a mild reduction in shoot formation capacity, yet at medium concentrations, results comparable to those of the control group are attained. These findings suggest that boric acid can support the shoot formation capabilities of plants at suitable concentrations. Analysing the impact of sodium perborate application, it is observed that shoot counts experience a slight decline at low concentrations, while a significant increase is noted at both intermediate and high concentrations. These observations suggest that sodium perborate might enhance the shoot formation capacity of plants, particularly under conditions of elevated stress. Upon examining the effects of sodium metaborate treatment, reductions in shoot counts are seen at both low and medium concentrations, with a more pronounced decrease at high concentrations. These results indicate that high concentrations of sodium metaborate could adversely affect the shoot formation capacity of plants. When evaluating the influence of disodium octaborate treatment, it becomes apparent that reductions in shoot counts occur at

both low and high concentrations, while results closely resemble those of the control group at intermediate concentrations. These findings imply that disodium octaborate has the potential to support plant shoot formation at appropriate concentrations (Figure 5).

In vitro propagation of woody plants has some limitations due to genetic changes in cultures' growth and aging process. *In vitro* cultures are a widely used method to propagate important woody plant species (Kıvrak-Kıran et al., 2021) and cytokinin and/or cytokinin and auxin growth regulators are used at optimized ratios for culture initiation. Nutrient media that do not contain plant growth regulators or contain auxin-like plant growth regulators for rooting (George *et al*., 2007) and indole butyric acid (IBA) are often used to stimulate the roots of woody plants (Kataeva & Butenko, 1987). Micropropagation of woody plant species is crucial for enhancing forest yield by producing high-value commercial seedlings. Furthermore, mineral nutrition is a significant factor in inducing organogenic responses. This study aimed to investigate the impact of various boron salts on the organogenesis of meristems derived from *in vitro* cultured *L. orientalis* micro-shoots. Culture medium supplemented with different concentrations of different boron salts differentially affected the *in vitro* organogenic control of *L. orientalis*. Boron-induced callus formation in explants allows the initiation of well-developed micro-sprouts that can be used for bud development. Similarly, Brondani *et al*. (2012) evaluated the effects of calcium and boron on nodal segment regeneration from Eucalyptus grandis micro-shoots.

Murashige and Skoog (MS) medium supplemented with different concentrations of calcium and boron (Murashige & Skoog, 1962) was modified to induce regenerative responses in 45-day-old E. grandis nodal explants and after 60 days dry weight, fresh weight, fresh and dry weight percentages, relative dry weight, fresh weight, fresh and dry weight accumulated by explants, water content and relative substance content were evaluated. The culture medium supplemented with different concentrations of calcium and boron was found to affect the organogenic control of E. grandis *in vitro*. It is known that the physical and superstructure properties of cell walls are affected by boron deficiency (Findeklee & Goldbach, 1996). Also, when plants are grown with insufficient boron, boron accumulates mainly in the cell wall (Hu *et al*., 1996). Large amounts of boron are not required by plants, but if not supplied in required amounts it can cause critical plant growth problems. Lack of chlorosis due to boron deficiency, however, has similar toxicity effects with other microelements. Boron, together with calcium, is used in plant cell wall formation and required for plant cell division. Other roles of boron include carbohydrate metabolism, sugar translocation, potassium transport to the stoma, nitrogen metabolism, pollen germination, regulation of hormone levels and formation of certain proteins, regular functioning and growth of apical meristems, membrane function and structure, and nucleic acid synthesis (Liang *et al.*, 2011). In this study, four different boron salts (boric acid, sodium metaborate, sodium perborate and disodium octaborate) were used as boron microelement source at three different concentrations and compared with the control group 3 mg/L boric acid and 5 mg/L. While an increase in the number of stems was observed in 1 mg/L sodium perborate application, it was observed that 5 mg/L disodium octaborate application was also effective on stem elongation. In plants, borate salts and boric acid are actively transported into cells as undissociated boric acid when boron levels are insufficient. At higher soil concentrations, passive diffusion takes place. These compounds are then transported to leaves via the xylem, where they are deposited and remain as the water evaporates. However, they show little movement to other parts such as the stem and fruit, and are inert in the phloem (Lank & Wahl, 2014). Boron is an essential microelement for higher plant species with levels of interspecific variation demanded for maximum plant growth (Lovatt & Dugger, 1984). Studies on boron show that boron plays an important role in cell wall crosslinking, including complexing with specific pectin content (Loomis & Durst, 1992). Examination of the functions of borax and boric acid on Vicia faba and other plant species showed the effect of boron in plant

nutrition (Warrington, 1923). The first large-scale study of boron's functions on plant development was carried out where fifty plant species were selected and grown in sand cultures with normal nutrient solutions at different boron concentrations. The best plant growth, low plant damaging concentrations, deficiency symptoms, trace boron levels were recorded for each species. The majority of plants showed best growth up to 5 mg/L boron levels and approximately one-third of the plants grown at trace boron levels showed morphological signs of deficiency. It was concluded that the beneficial and harmful effects of boron salts overlap among plant species, and are therefore divided into three broad categories sensitive, semi-tolerant and tolerant (Eaton, 1944). Tolerant strains withstand high boron concentrations with little effect, and sensitive strains react strongly to too much or too little boron. In light of the results obtained from our study, it can be said that it is in the semi-tolerant category for *L. orientalis*. Many studies in the literature show that various variations are noticed in plants propagated by *in vitro* tissue culture techniques (Barret *et al*., 2006; Baranek *et al*., 2010). These variations, spontaneous in nature or caused by environmental factors, are induced by different stress conditions and biochemical compounds in *in vitro* cultures. Diversity arises from differentiated cells using multiple or single cells during embryogenesis and organogenesis, and *in vitro* cultural condition induces regulation of pre-existing variation expressions. Many of these variations pose a major problem for seedling producers. However, these changes are promising for future studies focusing on plant quality.

4.2. Effect of Boron Salts on Phenolic Substances of *Liquidambar orientalis* Plant

When the samples incubated in WPM medium containing four different boron salts at different concentrations were compared with the control group, it was determined that the best result in the pyrogallol content was in the 1mg/L application of sodium perborate salt. It has been determined that 3-4-Hydroxy benzoic acid, Catechin hydrate, Quercetin, Epicatechin and Gentisic acid are major in *Liquidambar orientalis* plant. As a result of the boron salts applied at different concentrations, it was observed that the salt applications other than the plants applied boric acid increased the amount of Gentisic acid. It was determined that the amount of phenolic substance decreased as the amount of salt applied increased on the changes on catechin hydrate. Among the boron salts applied to the *L. orientalis* plant, it was observed that the phenolic content increased significantly as a result of the application of sodium perborate and disodium octaborate salts at a concentration of 3mg/L. It was observed that the amount of phenolic phenolic substance increased when the amount of Quarcetin, another major phenolic, was applied at low concentration, and this amount decreased significantly as the amount increased. On the other hand, epicatechin, another major phenolic compound, application of boric acid, sodium perborate and sodium metaborate at a concentration of 1 mg/L increased the density of this compound, while the application of higher concentrations decreased the density of epicatechin. As a result of disodium octaborate salt application, it was observed that the concentration amount of 1mg/L and 3mg/L concentration increased the epicatechin density, while the application at 5 mg/L concentration decreased the amount of this compound (Table 1).

As a result of this study, it is seen that the *L. orientalis* plant has half tolerance to boron salt stress, while the applied boron salts concentration of 5mg/L begins to affect the plant negatively. In order to better understand the living conditions of the *L. orientalis* plant, it will be possible to transfer the plant to future generations by doing more studies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Muhammed Mustafa Polat: Investigation, Formal Analysis and Writing. **Ergun Kaya:** Investigation and Critical Reading. **Ibrahim Kivrak:** Methodology, Supervision, Validation and Writing.

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Research Article

Total polyphenols and antioxidant activity of yellow velvetleaf (*Limnocharis flava*) extract

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Abstract: An increase in free radicals can cause damage to cells and tissues in the body. This is caused by a lack of antioxidant ability in the body, so it requires antioxidants outside the body. One of the plants that can be used as a source of antioxidants is the yellow velvetleaf (*Limnocharis flava*), which has a class of polyphenolic compounds. The crude extract of this plant still has other components that are not included in the polyphenol compound. This study aimed to measure the total polyphenol and flavonoid contents as well as the antioxidant activity of the yellow velvetleaf plant (L. flava) before and after purification. The purification process is carried out using the solid-phase extraction method. The antioxidant activity was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The total polyphenol and flavonoid contents increase after the purification process. Therefore, the antioxidant activity of purified extract is also increased when compared to crude extract. These results show that the purification process successfully increased the levels of polyphenol compounds from the yellow velvetleaf plant and its antioxidant activity. Thus, the purified extract can be used as an alternative source of natural antioxidants and can be developed as a food supplement ingredient.

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1. INTRODUCTION

Free radicals are a group of compounds that have unpaired electrons in their outer orbitals and are naturally the result of biological processes in the body. Free radical compounds can also come from pollution, cigarette smoke, exposure to heavy and transition metals, pesticides, and industrial wastes (Phaniendra *et al*., 2015). Under normal conditions, the body's antioxidant defense system can reduce exposure to existing free radicals because it has primary antioxidants (endogenous antioxidants), such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Ighodaro & Akinloye, 2019). However, the body requires additional antioxidants that come from outside when the levels of free radicals exceed the antioxidants contained in the body. This type of antioxidant can be classified as exogenous

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antioxidants and can generally be obtained through functional foods or dietary supplements (Roehrs *et al*., 2011).

Yellow velvetleaf (*Limnocharis flava*) is an aquatic plant commonly found in South Sumatra, Indonesia. This plant contains some bioactive compounds, such as polyphenols and flavonoids (Jamila *et al*., 2021). This plant extract also shows antioxidant activity by scavenging free radicals using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method (Baehaki *et al*., 2019).

Phenolic compounds are classified as secondary metabolites commonly distributed in the kingdom of plants, with immense structures and functions. Polyphenol compounds are a source as natural antioxidant (Swallah *et al*., 2020). Since polyphenol extraction with organic solvent was obtained as a crude extract composed of other non-polyphenol components, such as sugar, lipid, and organic acids, a purification process is required to remove these compounds (Zeka *et al*., 2017). A solid-phase extraction (SPE) method was used for the purification process due to its simplicity, rapidity, and economics (Dai & Mumper, 2010). A previous study reported that purified polyphenol compounds exhibit stronger antioxidant activity than crude (unpurified) extract (Wang *et al*., 2019). Additionally, the purification process of polyphenol extract by the SPE method also increases the inhibition of HMG-CoA reductase activity (Sudirman *et al*., 2022). According to these conditions, we hypothesized that the purification process also showed different effects on the antioxidant activity of the yellow velvetleaf extract. Therefore, this study aimed to investigate the effect of the purification process on the polyphenol and flavonoid contents of yellow velvetleaf (*L. flava*) extract as well as its antioxidant activity.

2. MATERIAL and METHODS

2.1. Preparation and Extraction

The fresh yellow velvetleaf (*L. flava*) was collected and washed using tap water. The whole plant was used except the root. The plant was cut into pieces and dried in the oven at 45ºC for 24 hours. After the drying process, the plant was ground to obtain dried powder. The extraction process was conducted at 30ºC for 3 hours by the maceration method with stirring at 100 rpm by a magnetic stirrer. Whereas, 70% ethanol was used as a solvent (Sudirman *et al*., 2022). Briefly, 20 g of dried sample was put into an Erlenmeyer tube, which contains 200 mL of 70% ethanol. After 3 hours of maceration, the filtrate and residue were separated by filter paper (Whatman No. 42). The filtrate was kept in the new collection tube and the residue was extracted using a fresh solvent under the same conditions as the first extraction, for a total of five extractions. After the extraction process, the filtrates were mixed in a new collection tube and the solvent was evaporated by using a rotary vacuum evaporator at 40ºC to obtain paste form, and then dried by using a freeze dryer. The yield of extraction was calculated according to this formula:

$$\text{Yield (\%)} = \frac{\text{Extract weight (g)}}{\text{Dried sample (g)}} \text{ x } 100\%$$

2.2. Purification Process

The purification process was conducted using a solid phase extraction method (SPE) by using HyperSep Retain PEP Cartridges (Part. No. 60107-212, Thermofisher Scientific) according to the previous method (Sudirman *et al*., 2022). Briefly, the cartridge was washed with 2 mL of distilled water and 2 mL in the precondition step, respectively. Then, 2 mL of the crude extract (1 mg/10 mL) was pipetted into the cartridge and was eluded by using 2 mL of *n*-hexane and 2 mL of H_2SO_4 1 N, respectively. The cartridge was then washed with absolute methanol to collect the purified extract and it was dried by a freeze dryer to obtain extract powder.

2.3. Total Polyphenol and Flavonoid Contents Analysis

The total polyphenol content was analyzed using *Folin-Ciocalteu's* method according to the previous method (Chandra *et al*., 2014). Briefly, 0.2 mL of each extract (10 mg/mL, crude and purified extracts) was mixed with Folin-Ciocalteu's phenol reagent in a reaction tube and allowed to react for 5 minutes. After the reaction process, the solution was added with 8% sodium carbonate and a volume of up to 3 mL using distilled water. The mixture was reacted in a dark condition for 30 minutes. The supernatant was separated by centrifugation at 3,000 rpm for 30 minutes. The absorbance of the supernatant was measured by a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA) at 750 nm. Gallic acid was used as a standard to determine the total polyphenol compound and the total polyphenol content was calculated as mg gallic acid equivalent (GAE) per g of dried weight (mg GAE/g DW).

The total flavonoid content was analyzed by the aluminum chloride colorimetric method according to a previous study (Chandra *et al*., 2014). Briefly, 0.5 mL of extract solution (crude and purified extract) was reacted with 2% aluminum chloride (1:1) at room temperature for 60 minutes. After the reaction time, the absorbance was measured immediately by using a spectrophotometer (Genesys 150 ThermoScientific, Massachusetts, USA) at 420 nm. The quercetin was used as a standard to determine the total flavonoid compound and the total flavonoid content was calculated as mg quercetin equivalent (QE) per g of dried weight (mg QE/g DW).

2.4. Antioxidant Activity Assay

The antioxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the previous study (Chew *et al*., 2008). Briefly, the extracts (crude and purified) were diluted at serial concentrations (0 – 1,000 µg/mL). Then, the extract solution (1 mL) was mixed with 0.2 mM DPPH solution (1:1). The mixture was incubated at 37ºC for 30 minutes in dark conditions. The absorbance was measured immediately using a spectrophotometer at 517 nm. The percentage of radical scavenging or free radical inhibition was calculated by following this formula:

$$\text{Percentage (\%) of inhibition} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100\%$$

Whereas: Abs_{blank}, the absorbance at 517 nm without sample; Abs_{sample}, the absorbance at 517 nm with sample.

2.5. Data Analysis

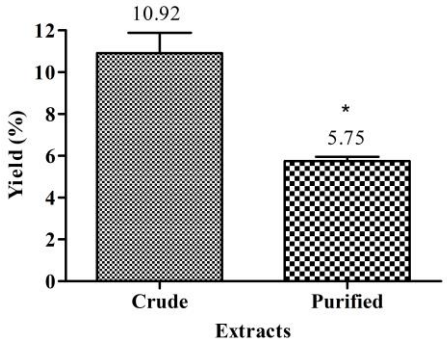
All the data were presented as mean ± standard deviation (SD). The data was analyzed using an independent t-test with SPSS 22.0 (IBM Corporation, Armonk, NY, USA). All graphics were produced using the GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA).

3. RESULTS

3.1. Extraction and Purification Yields

The yield of crude extract was about 10.92±3.59% and the yield was reduced to 5.75±0.25% after the purification process as shown in Figure 1.

Figure 1. The yield of crude and purified extracts of yellow velvetleaf (*L. flava*). Data was shown as mean ± SD (*n*=3). Significant difference at *p<0.05 vs crude extract.



3.2. Total Polyphenol and Flavonoid Contents

The total polyphenol content of yellow velvetleaf after purification significantly (p<0.05) increased when compared to crude extract (494.83 mg GAE/g DW and 379.25 mg GAE/g DW, respectively), as shown in Figure 2. Whereas, Figure 3 showed that the total flavonoids also significantly (p<0.05) increased after the purification process when compared to crude extract (280.98 mg QE/g DW and 177.61 mg QE/g DW, respectively).

Figure 2. The total polyphenol content of crude and purified extracts of yellow velvetleaf (*Limnocharis flava*). Data was shown as mean ± SD (*n*=3). Significant difference at *p<0.05 vs crude extract.

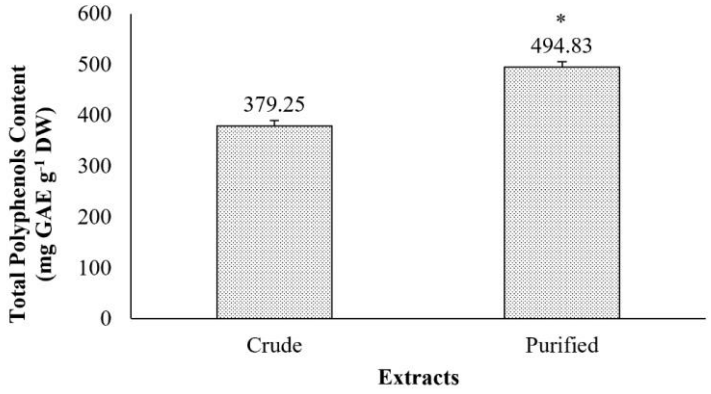
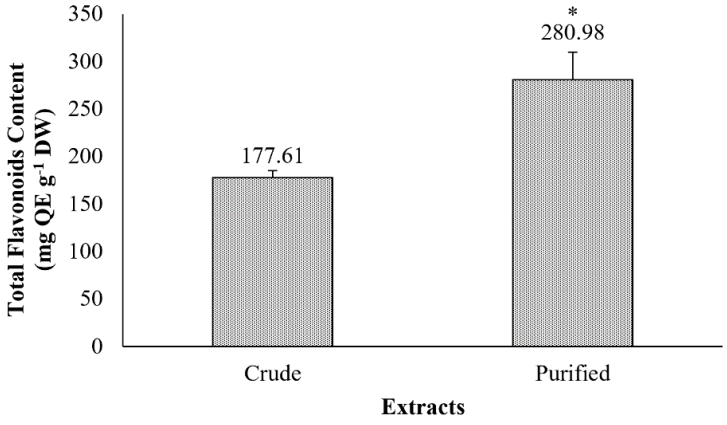


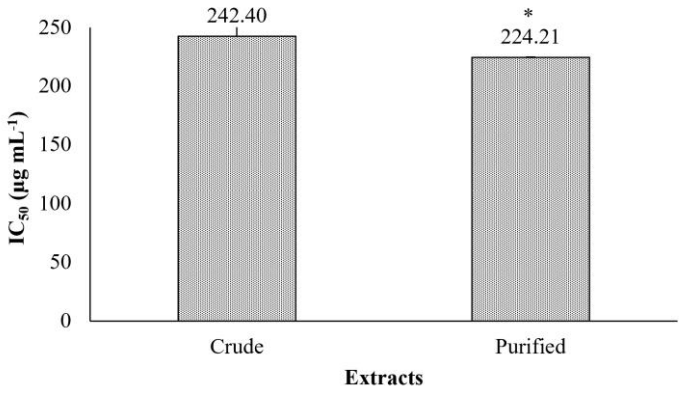
Figure 3. The total flavonoid content of crude and purified extracts of yellow velvetleaf (*Limnocharis flava*). Data was shown as mean ± SD (*n*=3). Significant difference at *p<0.05 vs crude extract.



3.3. Antioxidant Activity

The antioxidant activity of crude and purified extracts is shown in Figure 4. The antioxidant activity of purified extract significantly (p<0.05) increased when compared to crude extract (IC_{50} 224.21 µg/mL and 242.40 µg/mL, respectively).

Figure 4. The antioxidant activities of crude and purified extracts of yellow velvetleaf (*Limnocharis flava*). Data was shown as mean ± SD (n=3). Significant difference at *p<0.05 vs crude extract.



4. DISCUSSION and CONCLUSION

In this study, we successfully extracted polyphenol compounds from yellow velvetleaf (*Limnocharis flava*). The crude extract of polyphenol was purified using the solid-phase extraction method. The purified extract yield was reduced after the purification process (Figure 1). A previous study also reported that the crude extracts of *Quercus crassifolia* (Valencia-Avilés *et al*., 2018) and *Anacardium occidentale* (Nugroho *et al*., 2013) have a high yield when compared to purified extracts. This condition indicates that some unwanted compounds were successfully removed by the purification process. A solid-phase extraction method was used for the purification process due to its simplicity, rapidity, and economics. The purification process uses *n*-hexane solvent to remove nonpolar compounds such as lipoidal materials from the crude extract, whereas a low concentration of sulfuric acid was used to eliminate the non-phenolic and other organic compounds (Dai & Mumper, 2010; Sudirman *et al*., 2022).

The total polyphenol and flavonoid contents of the purified extract significantly increased compared to the crude extract after the purification process (Figure 2 and Figure 3). A previous study also reported that the total polyphenol content from water lettuce (*Pistia stratiotes*) leaf extract increased from 29.03 mg GAE/g dry sample to 65.63 mg GAE/g dry sample after the purification process. Whereas, the total flavonoid contents also increased after the purification process (crude extract, 27.58 mg QE/g dry sample; purified extract, 88.02 mg QE/g dry sample) (Sudirman *et al*., 2022). This condition indicated that the purified extract contained a concentration of polyphenols and flavonoids. The undesirable compounds were successfully removed from crude extract during the purification to obtain purified extract, such as lipoidal substances, non-phenolic compounds, and other organic compounds (Pérez-Magariño *et al*., 2008; Sudirman *et al*., 2022). A solid-phase extraction (SPE) purification method was used due to its being economic, simple, and rapid. The low concentrate of sulfuric acid was used to remove non-polyphenol polar compounds, such as organic acids and sugar as well as *n*-hexane was applied for the removal of non-polar from the crude extract (Pérez-Magariño *et al*., 2008).

The purification process successfully increases the antioxidant activity of the purified extract (Figure 4). A previous study also reported that the antioxidant activity of distiller grains increased after the purification process (Wang *et al*., 2019). There was high antioxidant activity in purified extract due to this extract also containing high-levels of polyphenol and flavonoid compounds. These compounds were categorized as secondary metabolites that are widely found

in plants and recognized as natural antioxidants (Dini & Grumetto, 2022). A previous study also reported that the high polyphenol content in the extract also shows high antioxidant activity (Yi & Wetzstein, 2011). Additionally, flavonoids (a group of polyphenols) are also reported as strong antioxidative agents (Panche *et al.*, 2016). The polyphenol compounds act as antioxidants by donating the hydrogen (H) atom to the free radicals (Matyas *et al*., 2019). Also, these compounds can reduce the oxidation reaction by transferring electrons (Lee *et al*., 2015).

Overall, in this present study, the polyphenol was successfully extracted from yellow velvetleaf (Limnocharis flava). The purification process increases the total polyphenol and flavonoid contents of the yellow velvetleaf extract. The antioxidant activity of this plant extract also increases after the purification process. Therefore, this extract potentially uses an alternative antioxidant agent and can be used as a food supplement ingredient.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Sabri Sudirman: Supervision and writing, editing and final approval. **Mey Arianti:** Methodology and formal analysis. **Gama Dian Nugroho:** Supervision and validation. **Sherly Ridhowati:** Formal analysis, and writing - original draft. **Puspa Ayu Pitayati**: Formal analysis and writing - original draft. **Miftahul Janna**: Methodology and formal analysis.

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Review Article

Bacteria-to-bacteria communication, Signaling Molecules: AHLs, AIPs and AI-2, I can't talk now matey, gone to pathogenesis!

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Abstract: How do these primitive tiny organisms' hierarchy operate? The answer is simple: by communicating. Yes, bacteria talk, and it's called: Quorum Sensing System (QS). QS is a phenomenon where bacteria tinychat and arrange their moves via the accumulation of signaling molecules. The cells respond to this stimulus when the cut-off value concentration of molecules is achieved. This phenomenon is widespreadly visible in the bacteria world. Bacteria never move solo and would need to gather up in critical mass because of secrete efficient toxins to be lethal. *N*-Acyl Homoserine Lactones (AHLs): Intercellular signaling molecules used by Gram-negative bacteria ($Gram^-$) to monitor their critical mass density in QS controlling of critic gene expression. AHL signals are synthesized by LuxI proteins. AHLs are vital interbacterial signaling molecules used by bacteria to check their dependent bacteria density. Auto-inducing peptides (AIPs): signaling molecules involved in intercellular communication in Gram-positive bacteria ($Gram^+$). Peptides are exported by dedicated systems, post-translationally modified and eventually sensed by other bacteria cells via membrane-located receptors that are part of a two-component system. AI-2 (Autoinducer-2): signaling molecule used by interspecies bacteria communication. This molecule chemically identified furanosylborate diester synthesized by LuxS proteins. Also, it's a universal signal because it carries out interspecies communication. The aim of this review is to summarize the AHL, AIPs, AI-2 bacterial signal molecules, QS systems target genes, effective in the procaryotic world, and the micro-social lifestyle of bacteria.

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1. INTRODUCTION

Tinychat comes true via *N*-Acyl Homoserine Lactones (AHL, *N*-AHLs or AHLs) signal molecules managed by a Quorum Sensing System (QS). QS is the coordination of bacterial group movies based on cell density. Thanks to this attitude, bacterial tinychats are established. As a result of tinychat, serious big consequences such as diseases occur (Parveen & Cornell, 2011; Winsberg, 2022). The properties that a real QS molecule should have, unlike a metabolite, are summarized as follows: I. Production of the QS molecule occurs at different phases of reproduction, under special conditions changes. II. The QS molecule agglomerates extracellularly and is detected whereby specific receptors. III. Accumulation of the QS molecule elicits a planned response when it reaches a critical threshold. IV. The cellular

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response elicited by the QS molecule is much broader than metabolizing or detoxifying the QS molecule. While many metabolites show the first three of these four features, the fourth feature is a feature that a QS molecule must have (Winzer *et al*., 2002).

Gram-negative bacteria ($Gram^-$) use AHL as their command language to coordinate population behavior during invasion and colonization of higher organisms. AHL is synthesized from precursors by a synthase protein, "LuxI" and once they have reached a certain cut-off value concentration, they interact with a transcriptional activating "LuxR" protein to induce target gene expression (Fuqua *et al*., 1996). So, QS operates that lets bacteria sense and answer target genes depending on their bacteria density. (Eberl *et al*., 1996; Pütz *et al*., 2022). In Gram-positive bacteria ($Gram^+$), the instrument of the communication system functions via Autoinducing Peptide (AIPs) (Sahreen *et al*., 2022). In interspecies, the instrument of communication is via Autoinducer-2 (AI-2) (Liu *et al*., 2022). Bacteria become more prepotent by communicating. They act simultaneously, perform critical gene expressions such as virulence, and perform gene transfer. They coordinate such multiple social behaviors through signaling molecules. In fact, different types of bacteria can communicate with each other thanks to signal molecules in crosstalk.

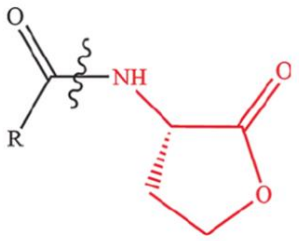
The aim of this review is to summarize the various AHL, AIPs, and AI-2 bacterial signal molecules currently available, QS system management prokaryotic world, and micro-social lifestyle of bacteria.

2. SIGNALING MOLECULES

2.1. AHL Molecules Structure

The main structural components of AHL (Figure 1) are hydrophilic sections of homoserine lactone ring (S-adenosylmethionine) and central amide group, as well as hydrophobic section strain-specific side hydrocarbon chain with varieties in length and level of oxygenation with a 3-oxo group. Acyl chain generally ranges from 4 to 18 carbons (Marin *et al*., 2007). In bacterial signaling, AHL is produced and released by bacteria cells. AHLs produced by different bacteria differ in the length of the R-group sidechain (Kumari, 2006). In QS, LuxI protein is synthesized by AHL. AHL can pass through the cell membrane down a gradient to environmental space. When molecule concentration reaches the threshold value, cognate LuxR protein binds to AHL. So, AHL directs target gene transcription (von Rad *et al*., 2008).

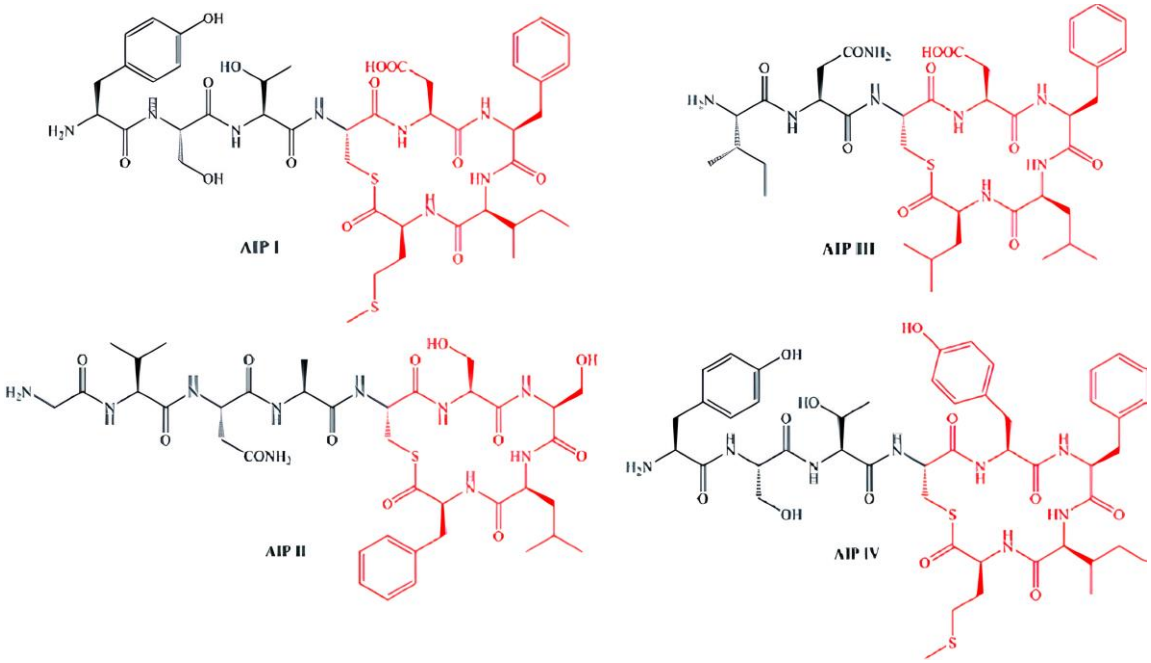
Figure 1. AHLs structure used by $Gram^-$ (Konai *et al*., 2018).



2.2. AIPs Peptide Signaling Molecules Structure

AIPs are characterized as AIP I-II-III-IV (Figure 2). Peptides may have differences in amino acid sequence; however, all of them possess an increased hydrophobicity from their N- to C-terminal end in peptide structure. At the end of the sequence to the C-terminal positions, AIPs are found to have amino acids with hydrophobic side chains (Mayville *et al*. 1999; MDowell *et al*. 2001; Yang *et al*. 2016). AIP linear peptide analogs or hydrolysis of the thioester moiety inactivates its functions molecule. Thioester macrocyclic ring is an ester moiety that was found to inactivate the signaling. Upon removal of N-terminal exocyclic structures, AIP loses signaling (Konai *et al*., 2018).

Figure 2. AIPs structure used by Gram^{+} (Konai *et al*., 2018).

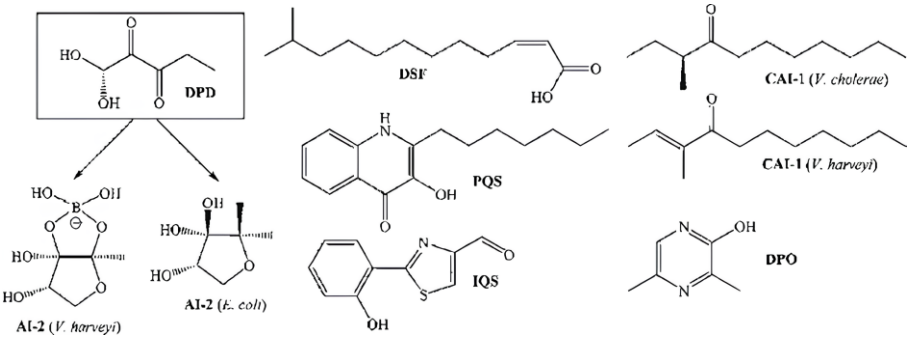


2.3. AI-2 Molecules Structure

AI-2 is a set of interconverting molecules derived from 4,5-dihydroxy 2,3- pentanedione (DPD) (Figure 3) (Qiao *et al*., 2022). LuxS is a synthase enzyme of DPD (Pereira *et al*. 2013). AI-2 synthesis is start with SAM (Federle & Bassler 2003). Different forms of DPD act as AI-2 for interbacteria. AI-2's chemical nature structure is the same for many bacteria species. This suggests that AI-2 is used for inter-species communication (crosstalk) to detect other bacteria (Miller *et al*. 2004). In AI-2-based QS, signal receive, and transduction are conducted by a two-component pathway. The first component consists of LuxP and LuxQ while the second component constitutes phosphotransferase LuxU and cytoplasmic response LuxO. There exist LuxPQ complexes with symmetric heterotetramer in AI-2. Upon binding with AI-2, the tetramer undergoes a change. This change prevents the phosphorylation of the cytoplasmic proteins, LuxU and LuxO, which terminates the expression of genes. Finally, LuxR is produced (Konai *et al*., 2018).

Significant works on the three-dimensional structures of proteins involved in QS first came into light in 2001 by determining the crystal structures of three LuxS orthologs with the help of X-ray crystallography. This was closely followed by determination of the crystal structure of the receptor LuxP of *Vibrio harveyi* with its inducer AI-2 (which is one of the few biomolecules containing boron) bound to it. AI-2 signalling is conserved among many bacterial species, including *Escherichia coli*, the model organism (Lewis *et al*., 2001; Chen *et al*., 2002). A database of QS peptides is existing "Quorumpeps" (Wynendaele *et al*., 2013; Wynendaele *et al*., 2015).

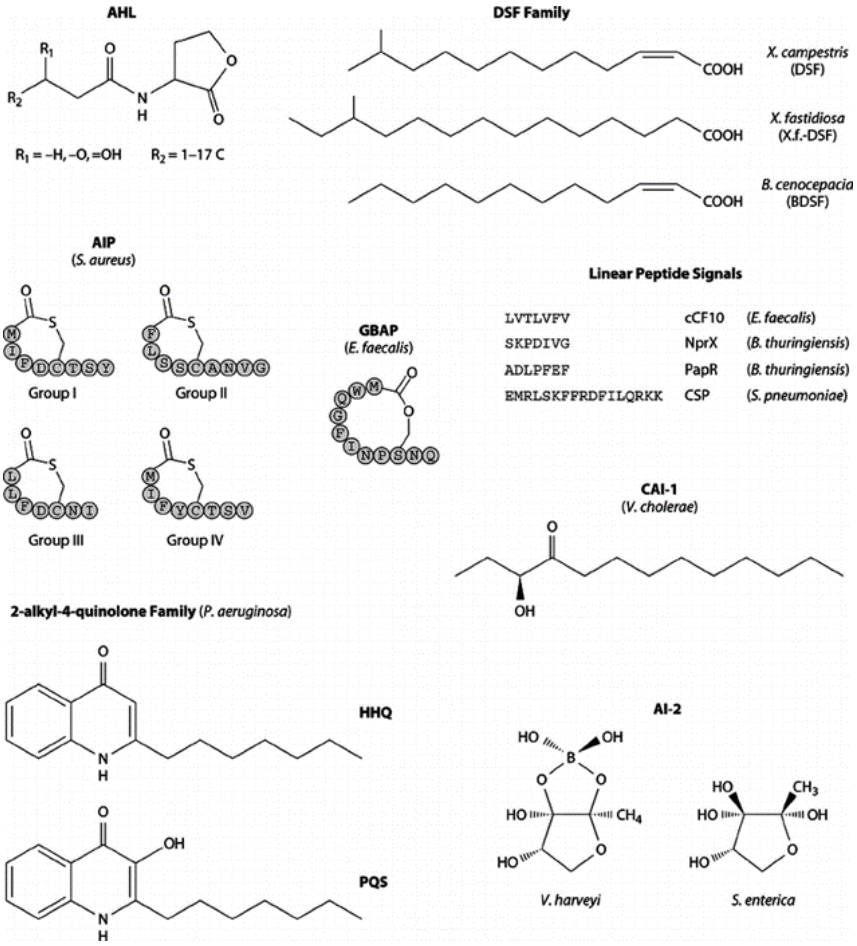
Figure 3. AI-2 and other signal molecules used by bacteria (Qiao *et al*., 2022).



3. COMMUNICATION INSTRUMENTS IN BACTERIA

Briefly, AHL is produced by LuxI-type and by LuxR-type in Gram. The second mechanism involves AIPs in Gram$^+$ which are sensed by two-component systems. The final system is the AI-2 system which is common in both Gram$^-$ and Gram$^+$ (Figure 4) (Brackman & Coeny, 2015). AIPs are often integral elements of a histidine kinase two-component signal transduction system. In this system, extracellular proteases process the secreted precursor-AIP into mature AIP. Kinase phosphorylates regulate gene transcription. This is called a two-component system (Rutherford & Bassler, 2012). AHLs are important "messenger" molecules involved in cellular communication of Gram$^-$ (Rutherford & Bassler, 2012). Usually, AHL does not need additional processing and binds directly to transcription factors to regulate gene expression (Bassler, 1999). Communication instruments in bacteria are shown in Table 1.

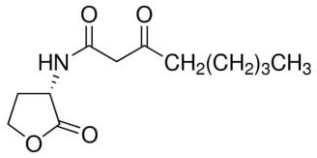
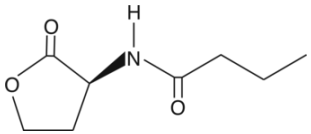
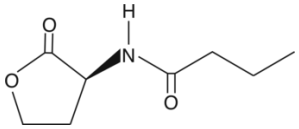
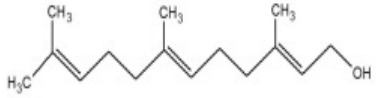
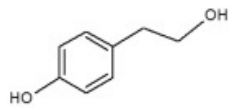
Figure 4. General structures of the selected QS signal molecules (LaSarre & Federle, 2013).

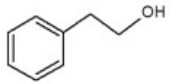
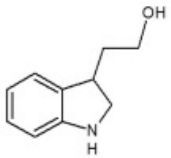
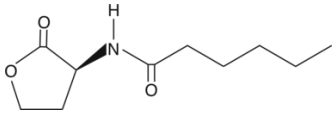
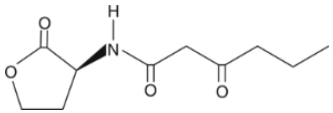
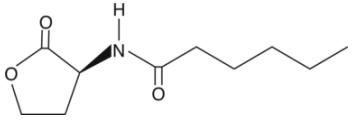
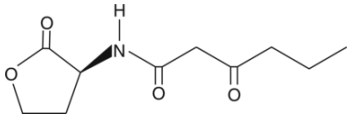


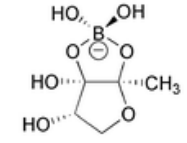
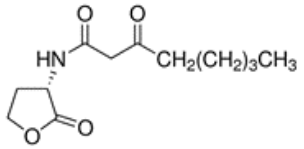
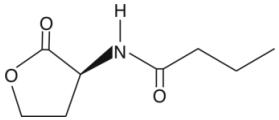
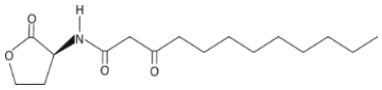
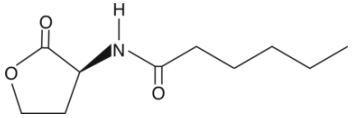
4. DISCOVERY of AHL, AIPs, AI-2

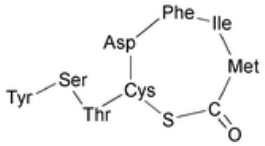
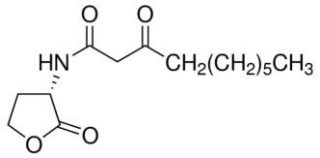
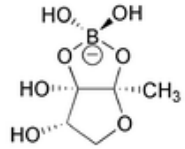
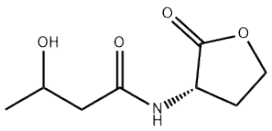
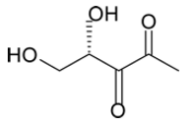
Previously pathogenic bacteria were tested against 2 AHL monitor bacteria (*Agrobacterium tumefaciens* NT1 and *Chromobacterium violaceum* CV026) in a well-diffusion assay (Bruhn *et al*., 2005). However, currently for AHL extraction problem is components present in bacterial culture supernatants. Components are cell growth media and extracellular products produced by bacteria. To reduce extracellular products, a stationary bacteria growth phase is recommended at extraction. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used methods to isolate AHL (Huang et al., 2020). Several methods have been developed for purification, detection and quantification of the AHLs. Methods have been developed for the detection of tinychat molecules. These methods are biosensors of HSLs, Thin-layer chromatography (TLC) (Laj *et al*., 2022), Radiolabeled assay (Schaefer *et al*., 2018), High-performance liquid chromatography (HPLC) (Bao *et al*., 2022), Colorimetry (Jin *et al*., 2020). For identification of AHL spectroscopic properties have been widely used to the characterization of QS molecule structures. Spectroscopic include Mass Spectrometry (MS) (Rosario *et al*., 2022), MS/MS, nuclear magnetic resonance spectroscopy (NMR) (Sundar *et al*., 2022), and infrared spectroscopy (IR) (Deepa *et al*., 2022). These methods are HPLC-MS (High-performance liquid chromatography-Mass spectrometry) and GC-MS (Gas chromatography-Mass spectrometry), Nano-LC-MS/MS (Frommberger *et al*., 2004), At-line coupling of UPLC (Ultra-high-pressure liquid chromatography) to chip-elctrospray-FTICR-MS (Fourier-transform ion-cyclotron resonance mass spectrometry) (Li *et al*., 2007a), Capillary zone electrophoresis mass spectrometry (CZE–MS), Nuclear magnetic resonance (NMR) (Bainton *et al*., 1992), IR spectrometry (IR) (Wang *et al*., 2011).

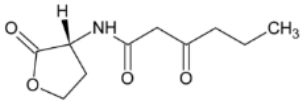
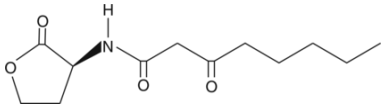
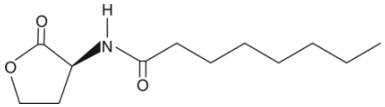
Table 1. Bacteria possessing QS system target genes, signal molecules, structure, and function in talking via chemical languages.

Strain	Gram* staining	Signal molecules	Structure	QS system target genes	Function	Reference
Agrobacterium tumefaciens	Gram$^-$	*N*-(3-oxooctanoyl)-L-homoserine lactone (OOHL)		*traR-traI*	Factors for conjugal Ti plasmids transfer	Zavilgelsky & Manukhov, (2001)
Aeromonas hydrophila	Gram$^-$	*N*-butanoyl-L-homoserine lactone (C_4-HSL, BHL)		*luxI-luxR* *AhyI-AhyR*	Virulence, biofilm, hemolysis	Filik, 2020
Aeromonas salmonicidae	Gram$^-$	C_4-HSL, BHL		*luxI-luxR* *AsaI-AsaR*	Exoprotease production	Miller & Bassler, (2001)
Bacillus subtilis	Gram$^+$	Competence and Sporulation Stimulating Factor (CSF)	Glu-Arg-Gly-Met-Thr		CSF adsorption, serious membrane damage, gene expression	Huang *et al*. (2021)
Candida albicans	Yeast**	Farnesol Tyrosol	 	*LuxS*	Dimorphism, biofilm	Kim & Yeon, (2018)

		Phenylethanol Tryptophol	 			
Chromobacterium violaceum	Gram$^-$	*N*-hexanoyl-L-homoserine lactone (C_6-HSL, HHL)		*luxI-luxR* *CviI/CviR*	Violacein pigment production	McClean *et al*., (1997)
Enterobacter agglomerans	Gram$^-$	3-oxo-C_6-HSL		*luxI-luxR* *EagI/EagR*	Pheromone production	Swift *et al*,. 1993
Erwinia carotovora	Gram$^-$	C_6-HSL, HHL *N*-(β-ketocaproyl)-L-Homoserine lactone (3-Oxo-C_6-HSL)	 	*luxI-luxR*	Carbapenem antibiotic production Virulence	Zavilgelsky & Manukhov, 2001 Burr *et al*., 2006

E. coli	Gram$^-$	AI-2		*LuxS*	Cell division, chromosome replication, cell signaling, translational modification, pathogenesis mechanismsprotein	Soni *et al*., 2007
		3OC$_8$HSL		NA (Not applicable) - SdiA	Motility, acid resistance	Soares & Ahmer 2011
Pseudomonas aeruginosa	Gram$^-$	C$_4$-HSL, BHL		*rhlI-rhlR*	Virulence, secondary metabolites (rhamnolipids)	Zavilgelsky & Manukhov, 2001
		N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL, 3OC$_{12}$-HSL)		*lasI-lasR*	Virulence (toxin A, elastase)	
Pseudomonas aureofaciens	Gram$^-$	C$_6$-HSL, HHL		*LuxI-LuxR PhzI-PhzR*	Produces of exoproducts, including protease, phenazines, phenazine antibiotic biosynthesis	Zhang & Pierson, 2001

Staphylococcus aureus	Gram^{+}	Oligopeptide Autoinducers Peptides (AIPs)		*Agr*	Virulence	Malone *et al*., 2007
Vibrio anguillarum	Gram^{-}	3-oxo-C_{10}-HSL (ODHL)		*LuxI-LuxR* *VanI/VanR*	Bioluminescence Violacein pigment production	Milton *et al*. 1997
Vibrio harveyi	Gram^{-}	*N*-octanoyl-L-homoserine lactone (OHL), Autoinducer-2 (AI-2) *N*-(3-hydroxybutanoyl)-L-homoserine lactone (HBHL), AI-1 4,5-dihydroxypentane-2,3-dione (DPD)	  	*ainS-ainR* *luxL-luxM* *luxR-luxN* *luxS-luxQ*	Bioluminescence	Geske, 2008

Vibrio fischeri	Gram$^-$	*N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), AI-1		*luxI-luxR*	Bioluminescence	Zavilgelsky & Manukhov, 2001
Yersinia ruckeri	Gram$^-$	OOHL OHL	 	*luxI-luxR*	Virulence	Bruhn *et al*., 2005

*Gram staining is an assay applied only to bacteria.
***C. albicans* is yeast and has nothing to do with gram stain.

5. DISCUSSION and CONCLUSION

Interbacterial communication systems based on signaling molecules have been determined in pathogenic $Gram^-$ and $Gram^+$ causing tough problems. The ability to mark bacterial communication at single-cell level in situ enables exploration of interbacterial communication, intercellular signal transduction, and QS, and it gives us a tremendous potential for studying the efficiency of communication systems in complex virulence systems scenarios (Andersen *et al*., 2001). Many species of microorganisms exhibit a social behavior.

QS in prokaryotic biology refers to the ability of bacteria to sense information from other cells in the population when they reach a critical concentration. They communicate with each other through signal molecules they have produced, monitor whether they have reached a certain majority, and trigger critical gene expressions such as synthesis of virulence as soon as they reach a sufficient majority. Thus, by not stimulating the host's immune system prematurely, it creates a successful infection process (Thirunavukkarasu *et al*., 2023).

Researchers have established “SigMol” (http://bioinfo.imtech.res.in/manojk/sigmol), a specialized repository of molecules in procaryotes. SigMol harbors information on QSSMs pertaining to different signaling systems namely AHL, AIPs, AI-2, and others. The database consists of 1382 entries of 182 peerless signal molecules from 215 microorganisms. SigMol encompasses biological (genes etc.) and chemical (IUPAC name, SMILES and structure etc.) properties of molecules (Rajput *et al*., 2016).

By secreting chemical toxins, all bacteria by themselves would receive the signals and answer them by activating the transcription of virulent genes and changing their social behavior to become highly pathogenic. Bacteria possess an extraordinary repertoire for interbacterial communication and social movements.

The discovery of communication molecules in the bacterial world attracts attention to fish disease control. Destroying AHL signal molecules, which are communication instruments, before they can occur the disease, brings up the concept of early diagnosis and in this case, it is aimed to era in prophylaxis. So, explaining to molecules in all detail will be an important step in diagnosis.

The similarities between the signals used by bacteria and artificial neural networks are striking. Based on the finding that bacteria have many of the properties of a neural network, bacteria may have a low level of intelligence. Researchers have stated that billions of bacteria collectively carry out the same command in their experiment by putting bacteria into communication with each other with the program loaded in their DNA. Scientists state that billions of bacteria that can communicate with each other can be managed at the same time and directed to certain tasks. They point out that in the future, smart biological devices will also be reflected in daily life.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nurdan Filik: Investigation, conceptualized the review articles. Also, a literature review. **Fethi Filik**: The draft preparation and involved in the drafting and edition. Also, a literature review. The drafts were critically discussed and revised by all authors.

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Research Article

Investigation of electronic and optical properties of nano-Ag produced from heterocyclic compounds by green chemistry reactions

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Abstract: The synthesis of nanoparticles using biological molecules has become one of the current research areas due to the high toxic content, poor stability, and expensive production technologies of nanoparticles synthesized by physical and chemical technologies. With the approach called green synthesis, nanoparticles that do not contain toxic substances have been produced in a method that does not harm the environment and human health. The number of polyphenol compounds in the ethyl alcohol/water extract of propolis collected from the Muğla-Türkiye region was determined. The synthesis and characterization of silver nanoparticles were carried out using the ethyl alcohol/water extract of propolis. The descriptions of the synthesized nanoparticles were made using ultraviolet-visible absorption and attenuated total reflection-Fourier transform infrared. Scanning electron microscopy, energy-dispersive X-ray, and X-ray diffraction methods were used for morphological examinations. Which polyphenol compound in the propolis content is effective in the synthesis of nanosilver particles was investigated with a Gaussian 16 package program. The electronic properties of the compounds were obtained by density functional theory using boundary orbitals theory, molecular electrostatic surface potential, and nonlinear optical properties. Epigallocatechin gallate, Kaempferol, and Quercetin are effective in obtaining nano-Ag and can be used as organic optical material in technology.

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1. INTRODUCTION

The word “nano”, whose etymological origin is based on Greek. A nanometer (nm) is a unit of length in the metric system that equals one billionth of a meter. It is a very small dimensional quantity that is beyond our perceptual limits. However, through nanoscience, we can understand the new behaviors of non-standard nanometric materials on the nanometer scale with the help of quantum theory. On the other hand, nanotechnology allows us to engineer matter on an atomic-molecular scale to unleash brand-new properties. Nanotechnology is a cutting-edge field that confidently pursues the creation of functional materials, devices, and systems that have the ability to manipulate, control, and produce physical, chemical, and biological

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phenomena at the atomic and molecular scale. Its precision and accuracy in achieving these goals make it an exciting and promising area of research. (Santhoshkumar *et al.,* 2017; Das *et al.*, 2009).

Metallic nanoparticles or metal nanoparticles have emerged as new terminology in the nanoparticular field over the past few years. Noble metals such as gold, silver, and platinum, which are biocompatible, are used for the synthesis of metal nanoparticles (Hojat *et al.,*2018). Different physical and chemical methods such as electrochemical changes, chemical reduction, and photochemical reduction are widely used for the preparation and stabilization of metal nanoparticles (Geethalakshmi R *et al.,*2012). One of the most remarkable metal nanoparticles is silver nanoparticles. Silver nanoparticles have several specific properties such as catalysis, magnetic and optical polarization, electrical conductivity, and microbial activity. Silver nanoparticles show a unique optical property because they support surface plasmons.

Numerous methods have been developed for the synthesis of silver nanoparticles in a solution medium. The most used methods are chemical precipitation, photochemical, electrochemical alcoholic reduction, polyol, solgel, microemulsion, etc. such as chemical methods (Bhattacharya *et al.,*2008). The use of chemical and physical methods in the synthesis of nanoparticles is very expensive and laborious and also leads to the presence of some toxic chemicals on the surface that harms the environment in applications (Siddiquee *et al.,*2020). Green synthesis used for metal nanoparticle synthesis is a biological approach using proteins, microorganisms, plants, or plant extracts, which has been proposed as an alternative to chemical methods. In the biosynthetic method, toxic chemicals are not used and the need for high pressure, energy, and temperature is not felt.

Propolis is a natural bee product that is used for many purposes in the hive, containing a mixture of wax, and resin, enriched with wax, pollen, and saliva secretions, which bees collect from the buds and bark of flowers and deciduous trees. Many scientific studies have shown that propolis has been used in the treatment of various diseases in traditional medicine since ancient times and has biological activities such as antimicrobial, antioxidant, antitumor, anti-inflammatory, and antiulcer. The structure and content of propolis vary according to the variety of plants that bees can reach. The physical properties of propolis, such as its chemical composition, also vary depending on the geographical structure and climate of the region where it is collected (Krell, 1996). The most important pharmacologically active components in propolis are flavonoids, flavanols, flavanones, alkaloids, steroids, terpenoids, and various phenolic and aromatics, followed by hydroxycinnamic acids such as caffeic, ferulic, acid, and caffeic acid phenyl esters (Petri *et al.,* 1988). It has been reported in previous studies that it is used in the synthesis of nanoparticles due to these large biomolecular compounds in bee propolis (Ramnath, 2017; Padil *et al.,* 2013). The proportions of biomolecular compounds in propolis vary considerably depending on the flower and tree structure in the region where the bee is located.

In this study, phenols contained in the ethanol extract of bee propolis collected from the province of Mugla-Türkiye were used as reducing and stabilizing agents for the biosynthesis of silver nanoparticles (P-AgNPs). The phenol and flavonoid contents of the ethyl alcohol extract of the propolis we have were analyzed. After determining the component amounts of the substances in propolis, computer calculations were used to understand which substance was effective in the synthesis of silver nanoparticles. The resulting nanoparticles were further characterized by UV-Vis, FTIR, EDX, SEM, and XRD analysis.

Density Functional Theory (DFT) is a very important method in fundamental sciences such as physics, chemistry, and biology since it gives a lot of information about the electronic structure of materials (Van Mourik *et al.,* 2014). In addition, the development of computer performance in recent years has led to the use of this method more effectively. Thus, it enabled

more experimental and computational studies to be carried out compared to 15-20 years ago. DFT calculations provide structural, force, and total energy information as well as system charge density. These features provide comparison and connection with experiments and identification of underlying mechanisms through the analysis of the electronic structure (Mason *et al.*, 2010).

2. MATERIAL and METHODS

2.1. Material

Silver nitrate ($AgNO_3$) and sodium hydroxide (NaOH) chemicals used to obtain silver nanoparticles were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All reagents and solvents used in antioxidant activity determinations are of analytical purity and used Folin-Ciocalteu reagent Sigma-Aldrich Chemie, Munich, Germany), TPTZ (tripridyltriazine) Merck (Darmstadt, Germany), Trolox (6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid) from Sigma Aldrich and HCl, ethanol from Merck (Darmstadt, Germany). Shaker Heidolph MR HEI-Standard (Schwabach, Germany) mixer Vortex Mixer Labnet VX100, MO BIO Laboratories, Inc. (NJ, USA) instruments were used for extraction. In addition, Spectro UV-Vis Double Beam PC LaboMed Inc., (Los Angeles, CA, USA) was preferred in UV-VIS spectrophotometer for antioxidant activity measurements. Rotary evaporator with hood IKA®-Werke, RV 05 Basic (Staufen, Germany) was used.

2.2. Preparation of Propolis Extract

Approximately 5 g of propolis collected from the region was taken and kept in a shaker (Heidolph Shaker, Schwabach. Germany) for 24 hours for the preparation of ethanol/water (1:1 v/v) extracts. In order to purify the solid particles in the solution, filtration was carried out using ordinary filter paper first and then blue band filter paper. Concentrations were determined by adjusting the final volumes. Stock solutions were kept at +4^{o}C until the analysis period.

2.3. The Total Amount of Phenolic Substance

The total phenolic content of ethanol/water extracts of crude propolis samples was determined according to the method developed by Slinkard and Singleton (Slinkard *et al.*, 1977). For this purpose, a standard calibration curve was prepared using the gallic acid standard. For this, different concentrations of gallic acid (500; 250; 125; 62.5; 31.25, and 15.625 µg/mL) were prepared and read at 760 nm by pipetting and incubating with Folin-Ciocalteu reagent. Then, the absorbance values against the concentrations were plotted. The total phenolic substance (TPM) content of the propolis extracts was found according to the graph drawn, and the amount of phenolic substance in the original sample was calculated as mg GAE (Gallic acid equivalent)/100 g propolis by using the dilution factors.

2.4. The total Amount of Flavonoid Substance

Total flavonoid content analysis was performed according to the method of Fukumoto and Mazza (Fukumoto *et al.*, 2000). Different concentrations of quercetin (1; 0.5; 0.25; 0.125; 0.0625; 0.03125 and 0.015625 mg/mL) were used to prepare the standard graph. The amount of flavonoid substance equivalent to quercetin was found according to the graph drawn with the absorbance values at 415nm against the concentration.

2.5. Determination of Total Antioxidant Activity

The FRAP method known as ferric reducing/antioxidant power (Fe(III)-TPTZ-2,4,6-tris(2-pyridly)-S-triazine) is reduced in the presence of antioxidants and the blue complex Fe(II) is reduced. The formation of TPTZ is based on the maximum absorbance of this complex at 593 nm. The FRAP reagent that gives the method its name was prepared daily. For this purpose, 100 µL of the sample was mixed with 3 mL of FRAP reagent (300 mM pH 3.6 acetate buffer,

10 mM TPTZ, and 20 mM $FeCl_3$ (10: 1: 1)) and absorbance was read at 593 nm after 4 minutes. Absorbances at 595 nm against the sample-free reference were read at 0 and 4 minutes. Different concentrations of $FeSO_4.7H_2O$ (31.25; 62.5; 125; 250; 500; 1000 μM) were used for calibration. The results were expressed as $FeSO_4.7H_2O$ equivalent antioxidant power (Benzie *et al.*,1999).

2.6. Synthesis of Silver Nanoparticle Using Propolis Extract

In this process, 10 ml of ethanol extract of propolis was added dropwise to 0.01 M $AgNO_3$ solution. After stirring for 10 minutes, pH was arranged to 10 by using 1 M NaOH and the solution was stirred until the colour changed from brown to black. Biosynthetic silver nanoparticles were centrifuged at 10.000 rpm for 20 minutes. The precipitated P-AgNPs were washed twice using water and ethanol to remove impurities present on them (Al-Fakeh *et al.*, 2021).

2.7. Characterization Techniques of P-AgNPs Derived from Propolis

Nanoparticles are typically characterized by their size, shape, surface area, and dispersion structure. It is a simple and useful analytical technique to measure the mass concentration of silver nanoparticles obtained by biosynthesis according to Beer's law. It is the technique used to measure the light absorbed and scattered by a sample (Tejamaya *et al.*, 2012).

20 mg of silver nanoparticles obtained by using propolis were taken and mixed with ethyl alcohol/water for half an hour in an ultrasonic bath to ensure complete mixing. It was filtered through the membrane filter in order to remove the aggregate products that may be in the solution containing nanoparticles. Scanning was performed between 200-800 nm using a quartz cell with ethyl alcohol as a reference (UV/Vis-1601, Shimadzu, Kanagawa, Japan).

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) is performed to identify functional groups on nanoparticles. To characterize the functional groups on the synthesized P-AgNPs, measurements were made with 4 cm^{-1} resolution and 50 scans (PerkinElmer Inc., Norwalk, CT, USA).

Scanning electron microscopy (SEM) based on electron microscopy is a technique used to characterize the morphology of nanoparticles through direct imaging. For SEM analysis, nanoparticles were mixed in ethanol for ten minutes in an ultrasonic bath to prevent aggregation in the medium. The resulting solution was coated with a conductive metal, a gold/palladium alloy, using a spray coater (Zeiss Supra 40 VP, Oberkochen).

EDX analysis is used to characterize nanoparticles synthesized by green synthesis technology. In this method, X-rays are emitted from the nanoparticles after being bombarded by an electron beam. Accordingly, the elemental composition of the nanoparticles can be determined (Quorum Q150R ES, Quorum Technologies Ltd, UK).

The X-ray diffraction (XRD) technique is used to examine the structural information of the metallic nanoparticle. Energetic X-rays can penetrate deep into materials and provide information about the structure. The broadening of the peaks in XRD confirms the formation of nano-sized particles. XRD analyzes (APD 2000 PRO GNR, Novara, Italy) of P-AgNPs were performed using CuK_α radiation and the wavelength of the X-Ray was 1.54059 Å'. For silver nanoparticles obtained from propolis, the scanning range was between $2\theta = 5\text{-}90^o$, and the measurement was carried out using an integration time of 3 seconds for each angle value. The XRD diffractogram obtained was evaluated semi-quantitatively according to the Match program on the computer connected to the diffractometer and according to the library of ICDD (International Center for Diffraction Data). The average particle size of the synthesized AgNPs was calculated by using the Debye–Scherrer equation.

$$D = \frac{k\lambda}{\beta cos\theta} \quad (1)$$

where k = shape factor (0.94); λ = X-ray wavelength (λ=1.5418 Å); β = full width at half maximum (FWHM) in radians; and θ = Bragg's angle.

2.8. Calculation Method

All computations were executed with density functional theory (DFT) at the B3LYP levels of theory with the Gaussian 16 Rev. B01 package (Frisch *et al.*, 2016) and GaussView 6.0.16 (Dennington *et al.*, 2016) was used for visualization of the structure. The compounds were optimized to get the global minimum by using B3LYP/SDD level. The SDD basis sets combine DZ and Stuttgart– Dresden ECP (relativistic effective core potential) basis sets. In our calculations, we tried common basis sets but because of the Ag^+ atoms, we did not succeed the optimizing the molecules. The SDD basis is selected for all molecules. The SDD basis set is the probable quality for the system related to heavy metals which are instrumentally recommended for heavy metals (Tsipis, 2014; Abegg *et al.*, 19741). The absorption spectrum and the excitation energies using the TD-DFT method and natural bond orbital (NBO) analysis in the gas phase, and non-linear optical (NLO) parameters were computed by using the optimized geometry data. The electronic properties and electronic surface potential (ESP) characteristics were achieved from the optimized geometry.

3. RESULTS

Polyphenols with phenolic character, which are found in propolis and produced as secondary metabolites, are in different types and concentrations are agents responsible for antioxidant capacity. Their determination as a total reveals the antioxidant character of the source, they are found in. In addition, the total phenolic substance in natural extracts is important to determine the number of hydroxyl groups that provide antioxidant activity.

The total amount of phenolic substances in propolis by the Folin (Singleton, 1965, Singleton *et al.*, 1999) method, which is based on the formation of a coloured complex with Folin Ciocalteu reagent, is given in Table 1, the total phenolic content of the studied propolis samples was found to be 34.32 mg GAE/g.

It is one of the most frequently used methods to measure the total antioxidant capacity of natural products and extracts. It was first developed by Oyaizu (Oyaizu, 1986) and later modified by Benzie and Strain (Benzie *et al.*, 1999). In addition, the method is simple, fast, and inexpensive compared to other methods applied to determine total antioxidant activity. It is also a method that gives healthy results and does not require any special equipment (Prior *et al.*, 2003). The trolox equivalent of FRAP activities was determined and the values found are given in Table 1. Accordingly, a high FRAP value indicates high antioxidant activity.

Table 1. Total phenolic substance, antioxidant activity, and flavonoid values of propolis samples collected from Mugla-Türkiye.

Sample	Total amount of polyphenol substance (mgGAE/g)	The total amount of flavonoid substance (mg QE/g)	Total antioxidant capacity (Ferric reducing/antioxidant power) FRAP
Liquid ethanolic propolis (s)	34.32±0.44 mgGAE/ ml	10.18±0.06 gGAE/ ml	222.85±1.67 mmol $FeSO_4.7\ H_2O$/ml

The determination of flavonoids is also called the aluminium chloride colorimetric method, the principle of this method is based on the formation of a stable acid complex of aluminium chloride with the 4-keto and C-3 or C-5 (or both) hydroxyl group of the flavonoids. The total

flavonoid content of ethanolic propolis extracts was found to be 10.18 mg KE/g, and the data obtained are summarized in Table 1.

Propolis, a natural product, is the general name of the sticky substance derived from plant resins by honey bees. Propolis contains various chemical compounds such as polyphenols (flavonoid aglycones, phenolic acids, and their esters, phenolic aldehydes, alcohols, and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds. More than 160 compounds have been identified in propolis samples, and these compounds vary according to the botanical and geographic origin of propolis. For example, the main chemical compounds of propolis obtained from poplar trees are flavonoid aglycones, hydroxycinnamic acids, and their esters, while propolis obtained from birch trees contains flavonoid aglycones, Baccharis spp. Carbon prenylated derivatives of p-coumaric acid can be given as important active compounds in propolis obtained from (Anklam, 1998).

In this study, the content and amount of polyphenolic and flavonoid compounds in propolis obtained from Muğla-Türkiye were determined according to the Folin-Ciocalteu method (Table 2).

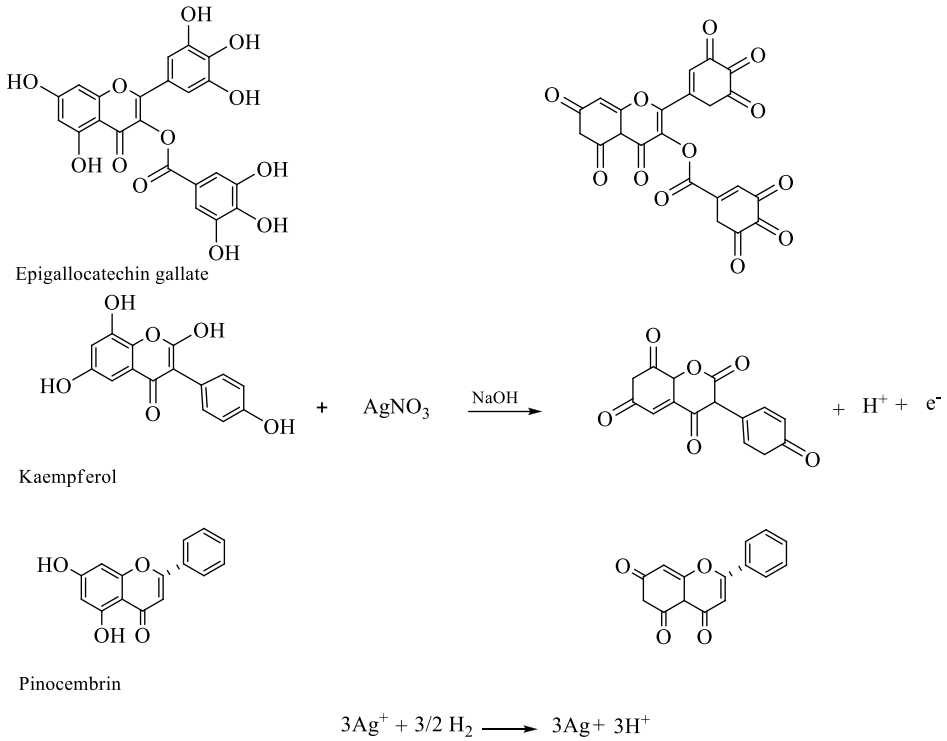
Table 2. Propolis content was obtained from Mugla-Türkiye the region.

Phenolic compounds determined in propolis	Amount (µg/ml)
3,4-Dimethoxycinnamic acid	142.16
Apigenin	287.01
Caffeic acid	292.55
Caffeic acid phenethyl ester	2102.26
Chrysin	419.76
Epigallocatechin gallate	24.34
Galangin	959.83
Gallic acid	30.28
Kaempferol	172.73
p-Coumaric acid	116.68
Naringenin	367.28
trans-Cinnamic acid	44.29
trans-Isoferulic acid	225.25
trans-Ferulic acid	86.00
Quercetin	468.02
Pinocembrin	958.08

The synthesis of silver nanoparticles was carried out as an environmentally friendly biosynthesis that does not harm nature, by using these substances in propolis as both reducing and stabilizing agents. It is a fast and simple method since the reaction time required for the formation of particles is shorter and a special system is not required for the reaction. The dark brown colour of propolis turning black as a result of the reaction with the ethanol extract is the first evidence of the formation of P-AgNPs.

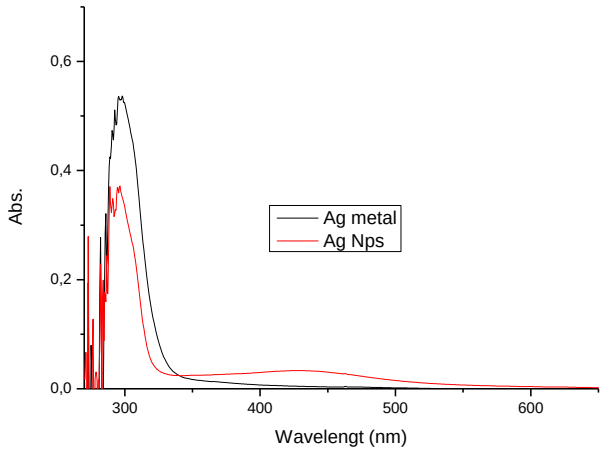
Polyphenols first form a silver complex with the silver ion, and then it is oxidized to the keto form with the release of free electrons and Ag^+ ions formed in the environment. Free electrons formed at the end of the reaction reduce Ag^+ ions to zero-valued silver (Ag^0) (Gautam *et al.*, 2007). At the same time, polyphenol compounds surround the formed silver metals and keep them in the form of nanoparticles (Figure 1).

Figure 1. The silver ions reduced by the phytochemical in the propolis extract are turned into nanoparticles by the stabilizing agent.



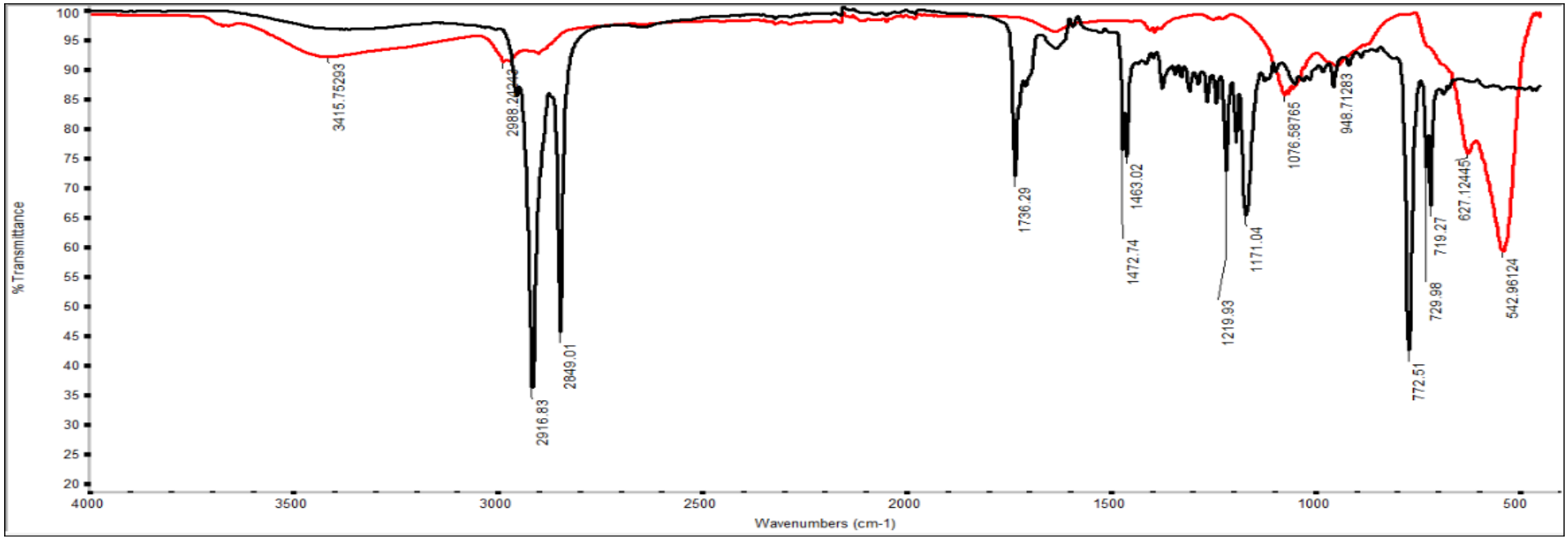
In order to determine the optical properties of silver nanoparticles obtained from the ethyl alcohol extract of propolis, a UV-visible absorption spectrum was taken between 200 and 800 nm. When the absorption spectrum given in Figure 2 is examined, it is seen that P-AgNPs have a maximum absorption at 335 nm (Jain S *et al.*, 2017).

Figure 2. Maximum absorbances of propolis extract and silver nanoparticles in UV visible spectrophotometer.



By comparing the propolis solid and the synthesized P-AgNPs, the FTIR spectrum confidently identifies the functional groups involved in the reaction (Figure 3). Between wavelengths 3552 – 4000 cm^{-1}, the broad peak corresponds to the stretching vibration of OH, while the double peak at 2915 cm^{-1} and 2849 cm^{-1} is due to the stretching vibration of CH. The occurrence of too many vibrations between 1500 - 1800 cm^{-1} is an indication of the intense carbonyl groups and the double bonds formed between C atoms and C, N elements. 1472 cm^{-1}, 1210 cm^{-1} are the C–O group of polyols (hydroxy flavonoids), aromatic ether peaks at 1171 cm^{-1}, and the peaks of alkene groups at 772 cm^{-1}.

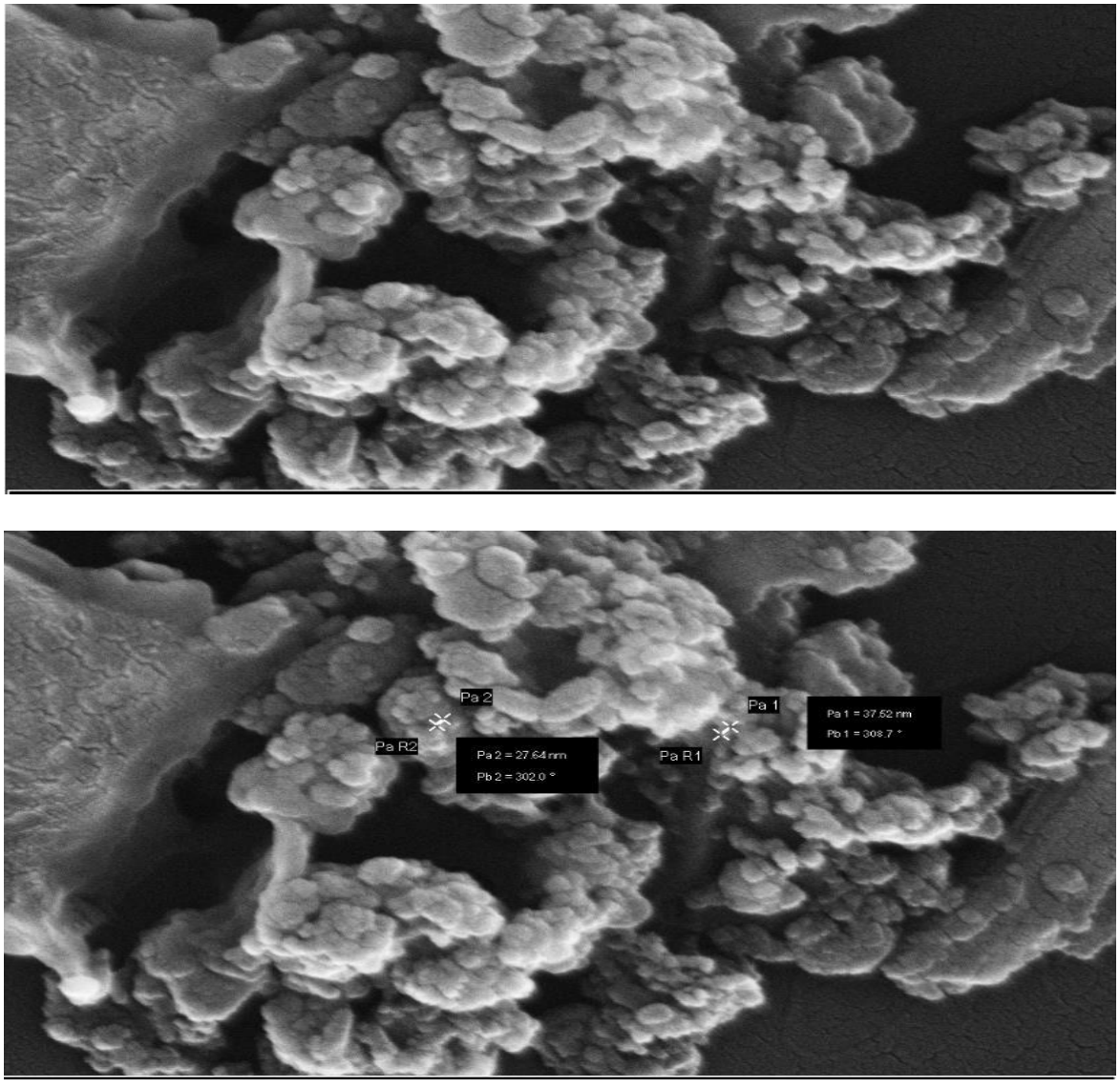
Figure 3. Functional groups attached in silver nanoparticle formation by FT-IR analysis **a.** FT-IR result of propolis extract (black line) **b.** FT-IR result after reaction result after interaction of propolis extract with silver nitrate (red line).



The bands formed in the spectrum of silver nanoparticles are similar to the bands formed in the spectrum of nanoparticles containing propolis. In the spectrum of propolis, the peaks at 2915 and 2849 cm^{-1} and the peaks at cm^{-1} could not be observed in the spectrum of nanoparticles. These peaks are suppressed by broad bands. Apart from this, all other peaks belonging to the propolis extract were also formed in the spectra of the nanoparticles (Lakshman Kumar *et al.*, 2016), the data of AgNPs synthesized by Echinochloa colona plant in the green synthesis, and characterization of AgNPs support our research.

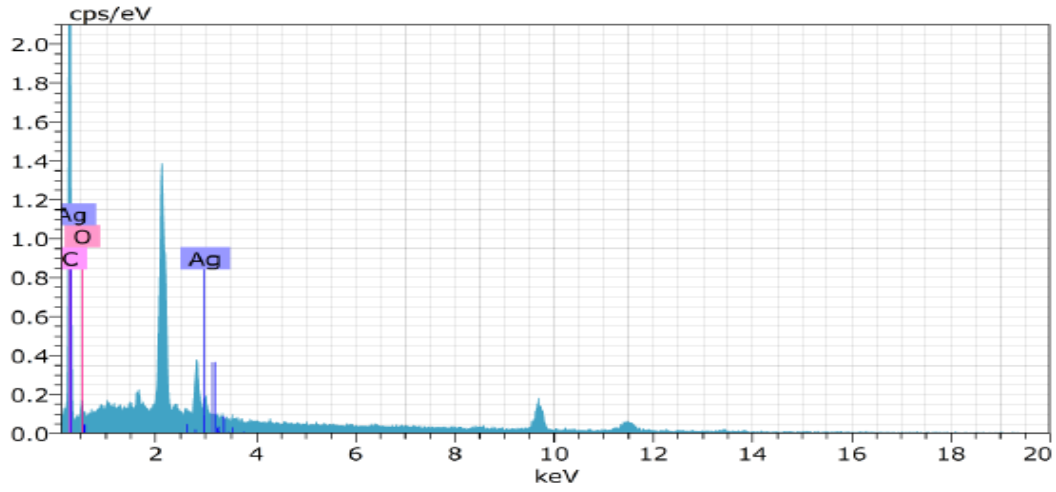
When the FTIR analysis of P-AgNPS is compared with the FTIR analysis of the ethanol extract of propolis, the change in the peaks of polyphenols (phenolics and flavonoids), carboxylic acids, and aromatic and carbonyl compounds is evidence of the formation of AgNPs and that these compounds act as capping agents in nanoparticles.

Figure 4. SEM image of silver nanoparticles produced using propolis extract.



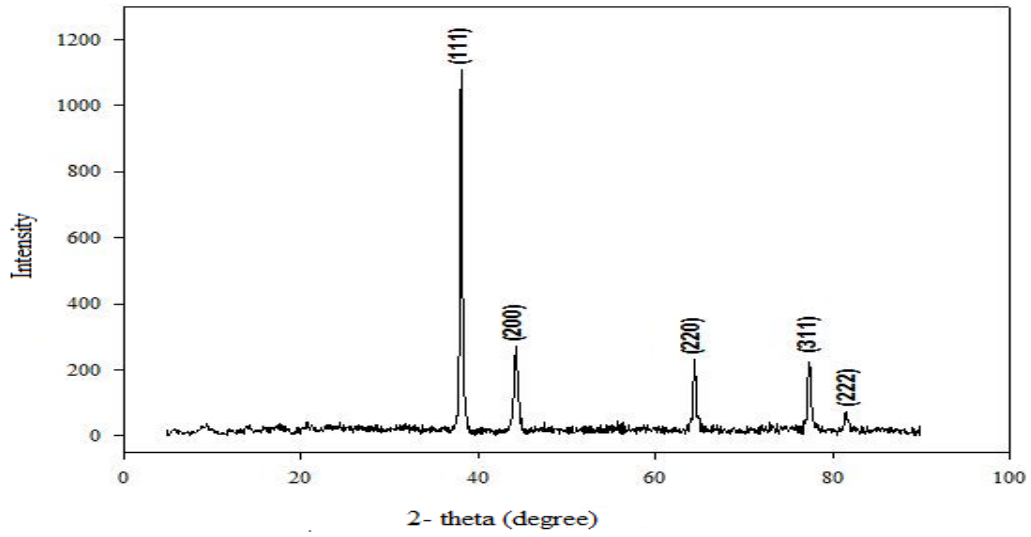
SEM is an important technique used to investigate the surface morphology of nanostructures. SEM images of the biosynthetic P-AgNPs sample are given in Figure 4. It is seen that Ag nanoparticles are randomly dispersed and have sizes ranging from 28 to 34 nm. Normally spherical Ag nanoparticles, which do not have a fully spherical structure in this study, are thought to result from the aggregation of two or more Ag nanoparticles during synthesis. This is in line with other studies in the literature (Dehvari *et al.*, 2018). In the energy-dispersive X-ray spectroscopy (EDX) analysis of the sample shown in Figure 5, 20.4% O and 79.6% Ag elements.

Figure 5. EDX spectroscopy shows the chemical composition of P-AgNPs.



(as atomic) were detected. EDX analyses of AgNPs showed over 79 % silver element as well as low proportions of carbon, oxygen, and nitrogen. Other than silver, other observed signals may have originated from biomolecules around AgNPs (Velammal *et al.*, 2016).

Figure 6. X-ray diffractogram/diffraction patterns of P-AgNPs synthesized using ethanol extract of propolis.

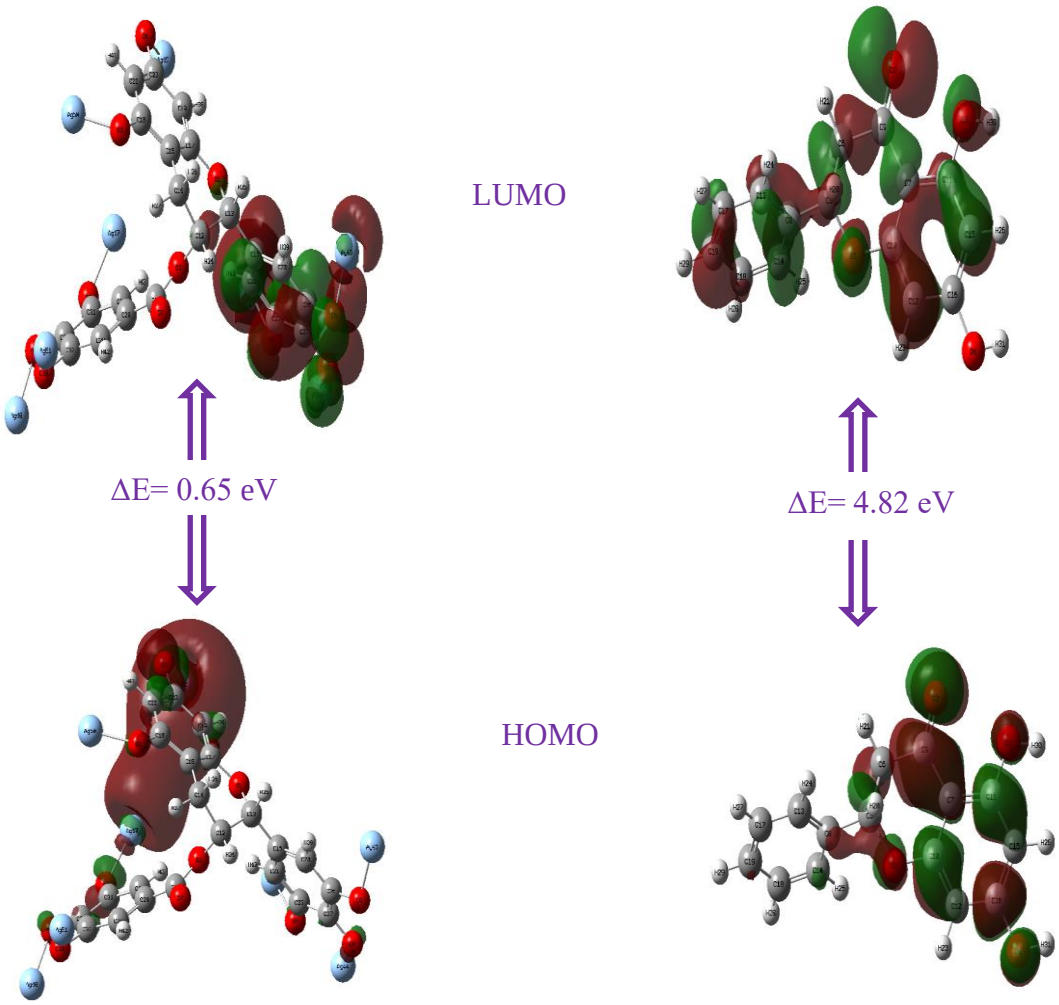


The powder XRD spectrum of the P-AgNPs sample is given in Figure 6 at angles of 27, 30, 34, 56, 37, 86, 46, 10, 64, 32 and 77.02 (2θ) originating from Ag nanoparticles; 2.67; 2.37; 1.97; Characteristic peaks corresponding to (222), (111), (200), (220) and (311) diffractions with distances of 1.44 and 1.24 Å were observed (JCPDS card no 04-0783). The positions and intensities of the obtained peaks indicate that Ag nanoparticles are face-centred cubic (Jasrotia *et al.*, 2020).

3.1. Electronic Properties

Quantum mechanical molecular orbital calculations give information about the transition state and active site for the reactions. The molecule which has a small energy difference between the HOMO and the LUMO orbitals represents the reactivity of the molecule, holding the two molecules in position for reaction, and also these molecules are called soft molecules (Stevens *et al.*, 2017).

Figure 7. Molecular orbitals diagram of Epigallocatechin gallate + Ag^+.



The value of the electronic descriptors calculated by Koopmans' theorem using the energy of the HOMO and the LUMO orbitals are given in Table 3. From Table 3, the polyphenols are more stable than the polyphenols binding with Ag^+ and also, and the most reactive molecule is the Epigallocatechin gallate + Ag^+ which has huge binding energy with the value of 0.65 eV and is also a soft molecule. Pinocembrin has low chemical reactivity and high kinetic stability with a value of 4.82 eV (Fukui, 1982).

The distribution of the molecular orbitals is very similar for all of the molecules shown in Figure 7 like pinocembrin except Epigallocatechin gallate + Ag^+. Due to the electron delocalization between C-C bonds (π*-π* interactions), as will be mentioned in Section Natural Bond Analysis, HOMO-LUMO orbitals show a distribution as shown in Figure 7.

Table 3. Electronic descriptors of the phenols.

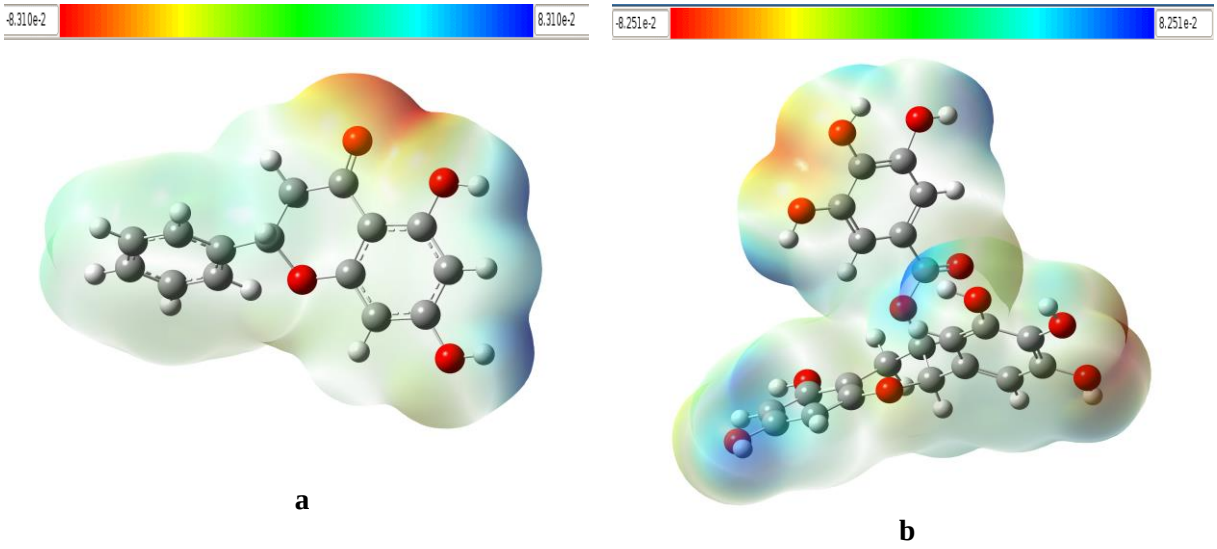
Phenols	Electronic descriptors of the phenols										Electronic descriptors at the end of the reaction of the phenols with Ag^+									
	E_{HOMO}	E_{LUMO}	ΔE	I	A	χ	η	s	μ	ω	E_{HOMO}	E_{LUMO}	ΔE	I	A	χ	η	s	μ	ω
3,4-Dimethoxycinnamic acid	-6.09	-2.10	3.99	6.09	2.1	4.10	1.99	0.50	-4.10	4.20	-5.79	-3.15	2.64	5.79	3.15	4.47	1.32	0.76	-4.47	7.57
Apigenin	-6.35	-2.36	3.99	6.35	2.36	4.36	1.99	0.50	-4.36	4.75	-4.76	-3.92	0.84	4.76	3.92	4.34	0.42	2.38	-4.34	22.42
Caffeic acid	-6.26	-2.21	4.05	6.26	2.21	4.24	2.03	0.49	-4.24	4.43	-5.36	-3.83	1.53	5.36	3.83	4.60	0.77	1.31	-4.60	13.80
Caffeic acid phenethyl ester	-6.16	-2.05	4.11	6.16	2.05	4.11	2.06	0.49	-4.11	4.10	-5.51	-3.99	1.52	5.51	3.99	4.75	0.76	1.32	-4.75	14.84
Chrysin	-6.44	-2.46	3.98	6.44	2.46	4.45	1.99	0.50	-4.45	4.96	-5.04	-3.70	1.34	5.04	3.7	4.37	0.67	1.49	-4.37	14.25
Epigallocatechin gallate	-5.91	-1.77	4.14	5.91	1.77	3.84	2.07	0.48	-3.84	3.56	-4.31	-3.66	0.65	4.31	3.66	3.99	0.33	3.08	-3.99	24.43
Galangin	-6.24	-2.37	3.87	6.24	2.37	4.31	1.94	0.52	-4.31	4.79	-4.65	-3.59	1.06	4.65	3.59	4.12	0.53	1.89	-4.12	16.01
Gallic acid	-6.49	-1.79	4.7	6.49	1.79	4.14	2.35	0.43	-4.14	3.65	-4.47	-3.55	0.92	4.47	3.55	4.01	0.46	2.17	-4.01	17.48
Kaempferol	-6.18	-2.30	3.88	6.18	2.3	4.24	1.94	0.52	-4.24	4.63	-4.53	-3.59	0.94	4.53	3.59	4.06	0.47	2.13	-4.06	17.54
p-Coumaric acid	-6.36	-2.19	4.17	6.36	2.19	4.28	2.09	0.48	-4.28	4.38	-5.25	-3.87	1.38	5.25	3.87	4.56	0.69	1.45	-4.56	15.07
Naringenin	-6.27	-1.64	4.63	6.27	1.64	3.96	2.32	0.43	-3.96	3.38	-5.00	-3.66	1.34	5	3.66	4.33	0.67	1.49	-4.33	13.99
trans-Cinnamic acid	-6.74	-2.30	4.44	6.74	2.3	4.52	2.22	0.45	-4.52	4.60	-6.34	-3.23	3.11	6.34	3.23	4.79	1.56	0.64	-4.79	7.36
trans-Isoferulic acid	-6.11	-2.14	3.97	6.11	2.14	4.13	1.99	0.50	-4.13	4.29	-5.07	-3.24	1.83	5.07	3.24	4.16	0.92	1.09	-4.16	9.43
trans-Ferulic acid	-6.26	-2.16	4.1	6.26	2.16	4.21	2.05	0.49	-4.21	4.32	-5.00	-3.35	1.65	5.00	3.35	4.18	0.83	1.21	-4.18	10.56
Quercetin	-6.17	-3.78	2.39	6.17	3.78	4.98	1.20	0.84	-4.98	10.36	-4.73	-3.78	0.95	4.73	3.78	4.26	0.48	2.11	-4.26	19.06
Pinocembrin	-6.46	-1.64	4.82	6.46	1.64	4.05	2.41	0.41	-4.05	3.40	-5.12	-3.73	1.39	5.12	3.73	4.43	0.70	1.44	-4.43	14.09

*The phenols were randomly numbered from 1 to 16.

3.2. Molecular Electrostatic Potential Analysis

Electrostatic surface potential (ESP) is a useful facility for cognizance of where the electron distribution effect is regnant. ESP maps demonstrate the positive, negative, and neutral electrostatic potential regions in different colors. Areas with a negative electrostatic surface associated with the electronegative atom pair are shown in red. As seen in Figure 8, the most reactive parts of the compound are around the O atoms with the highest electron density, while the stable parts are in the C-H regions with the lowest electron density.

Figure 8. ESP maps of **a.** Epigallocatechin gallate, **b.** Pinocembrin.



a **b**

3.3. Binding Energy

The binding energy of Ag^{+} is calculated as the given formula below;

$$RXO_nAg_n \rightarrow RXO_n^{-} + n(Ag^{0}) \quad (2)$$

As seen in Table 4, the binding energy of Ag^{+} to phenolics per atom is close to each other.

Table 4. The binding energies of Ag to the molecules (phenolics).

Num*	Phenolics	E_b (eV)	The Amount of phenolic compounds in propolis (mg/ml)
1	3,4-Dimethoxycinnamic acid	-21.4802	142.16
2	Apigenin	-23.5707	287.01
3	Caffeic acid	-24.0351	292.55
4	Caffeic acid phenethyl ester	-22.2552	2102.26
5	Chrysin	-22.3378	419.76
6	Epigallocatechin gallate	-28.8121	24.34
7	Galangin	-23.9137	959.83
8	Gallic acid	-26.5054	30.28
9	Kaempferol	-25.0347	172.73
10	p-Coumaric acid	-22.3906	116.68
11	Naringenin	-23.5788	367.28
12	trans-Cinnamic acid	-21.4298	44.29
13	trans-Isoferulic acid	-22.4824	225.25
14	trans-Ferulic acid	-22.4848	86.00
15	Quercetin	-26.5538	468.02
16	Pinocembrin	-22.9139	958.08

*The phenolics were randomly numbered from 1 to 16.

The molecular electrostatic potential has been used to foretell the reactive sides of the molecule and hydrogen bonding interactions as well as their potential use in biological recognition studies (Murray *et al.*, 1996; Scrocco *et al.*, 1978). Also, Figure 8 pointed out the electrostatic potential surfaces of Epigallocatechin gallate and Pinocembrin which have the highest and the lowest binding energy. The red regions show the nucleophilic and the blue regions show the electrophilic surfaces of the compounds.

3.4. Optical Properties

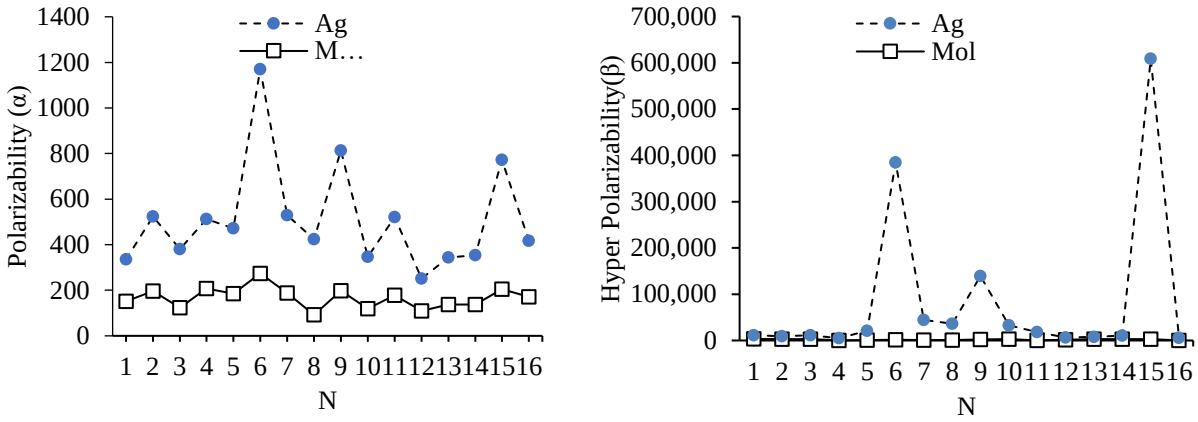
Polarizability is another significant characteristic of the electronic property of a molecule (Eşme *et al.*, 2014), DFT is used to compute the parameters such as; dipole moment (μ_t), the mean polarizability (<α>), and the total first static hyperpolarizability (β_t) for the title molecule. The parameters μ_t and <α> are connected with the first and higher-order derivatives of the electron density (Kleinman, 1962; Pipek *et al.*, 1989).

The magnitude of the molecular hyperpolarizability β is one of the key factors in the non-linear optical (NLO) system. The dipole moment (μ), mean polarizability (α), and first hyperpolarizability (β) values of the phenols have been computed at B3LYP/SDD level in the gas phase and solvent, respectively, and are presented in Table 5. The first static hyperpolarizability β values have been calculated as 12.541×10^{-30} in the solvent and 5.160×10^{-30} in the gas phase. The polarizability and the hyperpolarizability values have increased from the gas phase to the solvent phase. It can be said that this is due to stabilizing the polarized excited state of the molecule via solvent and/or hydrogen bonding and allowing the intramolecular charge transfer to occur more efficiently in the solvent. Usually, Urea is used as a prototypical molecule to determine the NLO properties of the molecular system (Prashanth *et al.*, 2015). The μ_t and β_t values for the title compound have been calculated as 5.0831 debye and 5.160×10^{-30} esu, whereas the references values for Urea are 1.3732 debye and 0.3728×10^{-30} esu, respectively. Thus, it appears that the total dipole moment and total first-order hyperpolarizability values of the molecule are greater than those of Urea.

Table 5. Dipole moment, Polarizability, and Hyper Polarizability values of the phenolic compounds in gas and in DMSO.

		Gas			DMSO		
Num	Compounds	Dipole Moment	Polarizability (α)	Hyper Polarizability (β)	Dipole Moment	Polarizability (α)	Hyper Polarizability (β)
		Mol	Mol	Mol	Ag	Ag	Ag
1	3,4-Dimethoxycinnamic acid	5.8	151.3	3419.7	1.9	184.3	7598.2
2	Apigenin	3.8	196.5	3023.8	3.7	327.6	6458.3
3	Caffeic acid	5.5	123.7	2704.4	3.2	257.1	8895.3
4	Caffeic acid phenethyl ester	5.1	207.3	207.3	6.1	305.0	4689.3
5	Chrysin	4.1	185.3	1194.5	8.3	286.5	19966.0
6	Epigallocatechin gallate	3.6	274.0	1707.8	6.4	896.3	382923.5
7	Galangin	5.3	188.1	799.6	8.4	340.5	43470.9
8	Gallic acid	2.6	92.2	1121.0	6.4	332.4	34829.2
9	Kaempferol	4.8	198.0	2445.0	4.8	615.1	137152.4
10	p-Coumaric Acid	3.8	119.6	2750.5	4.6	226.9	30222.6
11	Naringenin	4.0	178.0	562.5	3.9	343.0	17822.7
12	trans-Cinnamic acid	2.9	109.7	1396.7	1.7	141.7	4838.1
13	trans-Isoferulic acid	6.3	137.8	3237.3	5.2	206.1	4467.9
14	trans-Ferulic acid	4.4	137.0	2911.7	5.4	216.5	7665.1
15	Quercetin	6.0	204.3	3047.6	1.3	567.6	605591.2
16	Pinosembrin	2.9	171.3	568.4	4.8	245.3	5503.3

Figure 9. The alteration of the NLO properties to molecules in the extract.



*N: 16 molecules were found in the extract and these molecules were randomly numbered from 1 to 16.

The average polarizability and hyperpolarizability values of the phenols considered in the propolis content were calculated. After these molecules were bound with Ag, a very large increase in the values of molecules Epigallocatechin gallate, Kaempferol, and Quercetin was calculated. It can be said that after the binding of these molecules with Ag, the NLO properties increase even more. The results are illustrated in Figure 9.

3.5. Natural Bond Orbital Analysis

The charge transfer in the molecule in addition to intramolecular interaction is verified by natural bond orbital (NBO) analysis. To examine the interactions between occupied and virtual orbitals of a system, the DFT method has been used to foretell the delocalization of electrons (Karthick *et al.*, 2011; Arivazhagan *et al.*, 2012; Govindarajan *et al.*, 2012). NBO analysis (Weinhold, 1998), involving the deletion of matrix elements corresponding to the CO bonding/antibonding orbital as well as lone pair/ antibonding orbital interactions expands upon recent work that investigated stereoelectronic contributions to gauche stabilization through analysis of NBO $E^{(2)}$ transfer energies (Trindle *et al.*, 2003). The $E^{(2)}$ value indicates the interaction energy between electron acceptor and electron donors and this value is great for the addiction of electrons donation from donor to acceptor.

The results of the NBO analysis of the Epigallocatechin gallate and its compound binding with Ag are obtained at B3LYP/SDD level presented in Table 6 and Table 7, respectively. The hyper-conjugative σ→σ* interactions play an extremely significant role in the molecule and represent the weak departures from a strictly localized natural Lewis structure that constitutes the primary 'noncovalent' effects (Dege *et al.*, 2014).

Table 6 shows that the strongest stabilization energy in Epigallocatechin gallate is calculated as 370.09 kcal/mol between π* (C31 – C33) → π* (C28 - C30). Also, the interaction between π* (O7 - C24) → π* (C28 - C30) has 67.95 kcal/mol giving the structure a strong stabilization. Table 7 shows that the strongest stabilization energy in Epigallocatechin gallate + Ag^+ is calculated as 270.61 kcal/mol between π* (C20 - C26) → π* (C16 - C21). The interactions between π* (C18 - C22) → π* (C15 - C17) has 252.50 kcal/mol, π* (C31 – C33) → π* (C28 – C30) has 234.33 kcal/mol, π* (C19 – C23) → π* (C15 – C17) has 175.68 kcal/mol and π* (C25 – C27) → π* (C16 – C21) 164.47 kcal/mol also give the structure a strong stabilization.

Table 6. Remarkable stabilization interactions for the Epigallocatechin gallate.

Donor (i)	Type	ED/e	Acceptor (j)	Type	ED/e	$E^{(2)}$ (kcal/mol)
C15 - C17	π	1.68291	C15 - C17(π*)	π*	0.41195	1.57
			C18 - C22 (π*)	π*	0.40266	26.04
			C19 - C23(π*)	π*	0.40495	15.58
C16 - C20	π	1.71476	C21 - C25(π*)	π*	0.41169	17.42
			C26 - C27(π*)	π*	0.43158	22.19
C19 - C23	π	1.70563	C18 - C22 (π*)	π*	0.40266	25.73
			C15 - C17(π*)	π*	0.41195	24.66
			C18 - C22(π*)	π*	0.40266	14.59
C21 - C25	π	1.69289	C16 - C20(π*)	π*	0.41445	22.14
			C26 - C27(π*)	π*	0.43158	19.02
C26 - C27	π	1.62880	C16 - C20(π*)	π*	0.41445	19.44
			C21 - C25(π*)	π*	0.41169	23.30
C28 - C30	π	1.67577	O7 - C24 (π*)	π*	0.28410	26.40
			C29 - C32 (π*)	π*	0.38507	19.50
			C31 - C33 (π*)	π*	0.43423	21.52
C29 - C32	π	1.69421	C28 - C30 (π*)	π*	0.43818	19.74
			C31 - C33 (π*)	π*	0.43423	19.94
C31 - C33	π	1.60567	C28 - C30 (π*)	π*	0.43818	21.79
			C29 - C32 (π*)	π*	0.38507	21.81
O3	LP 2	1.88900	C18 - C22 (π*)	π*	0.40266	25.35
O4	LP 2	1.88882	C19 - C23 (π*)	π*	0.40495	25.78
O5	LP 2	1.90626	C21 - C25(π*)	π*	0.41169	21.67
O6	LP 2	1.88634	C26 - C27(π*)	π*	0.43158	24.53
O7	LP 2	1.85759	O2 - C24 (σ*)	$sp^{2.35}$	0.10635	32.22
			C24 - C28 (σ*)	$sp^{1.52}$	0.05845	15.00
O8	LP 2	1.87858	C26 - C27 (π*)	π*	0.43158	25.80
O9	LP 2	1.88497	C31 - C33(π*)	π*	0.43423	24.52
O10	LP 2	1.90539	C29 - C32 (π*)	π*	0.38507	22.06
O11	LP 2	1.86151	C31 - C33 (π*)	π*	0.43423	27.47
O7 - C24 (π*)	π*	0.28410	C28 - C30 (π*)	π*	0.43818	67.95
C31 - C33 (π*)	π*	0.43423	C28 - C30 (π*)	π*	0.43818	370.09

Table 7. Remarkable stabilization interactions for the Epigallocatechin gallate + Ag^{+}.

Donor (i)	Type	ED/e	Acceptor (j)	Type	ED/e	$E^{(2)}$ (kcal/mol)
C15 - C17	π	1.66666	C18 - C22	π*	0.43457	25.78
			C19 - C23	π*	0.44531	15.30
C16 - C21	π	1.69508	C20 - C26	π*	0.40437	16.24
			C25 - C27	π*	0.46256	20.78
C18 - C22	π	1.97078	C15 - C17	π*	0.41651	15.49
			C18 - C22	π*	0.43457	1.41
			C19 - C23	π*	0.44531	27.02
C19 - C23	π	1.97150	C15 - C17	π*	0.41651	25.02
			C18 - C22	π*	0.43457	13.69
C20 - C26	π	1.69804	C16 - C21	π*	0.41511	22.75
			C25 - C27	π*	0.46256	15.44
C25 - C27	π	1.53472	C16 - C21	π*	0.41511	22.40
			C20 - C26	π*	0.40437	18.52
C28 - C30	π	1.67799	O7 - C24	π*	0.30377	28.94
			C29 - C32	π*	0.38098	18.91
			C31 - C33	π*	0.02676	18.97
C29 - C32	π	1.72177	C28 - C30	π*	0.45446	18.65
			C31 - C33	π*	0.02676	16.54
C31 - C33	π	1.50483	C28 - C30	π*	0.45446	24.04
			C29 - C32	π*	0.38098	19.17
O1	LP(2)	1.87072	C15 - C17	π*	0.41651	22.64
O2	LP(2)	1.82316	O7 - C24	π*	0.30377	37.43
O3	LP(2)	1.85532	C18 - C22	π*	0.43457	19.23
O4	LP(2)	1.86700	C19 - C23	π*	0.44531	18.57
O6	LP(2)	1.84433	C20 - C26	π*	0.40437	15.34
O6	LP(3)	1.63801	C20 - C26	π*	0.44531	14.38
O7	LP(2)	1.86535	O2 - C24	$sp^{2.48}$	0.10338	31.38
			C12 - C13	$sp^{2.57}$	0.02505	0.79
			C24 - C28	$sp^{1.48}$	0.05627	13.97
O8	LP(2)	1.81321	C26 - C27	$sp^{1.88}$	0.06113	10.75
O8	LP(3)	1.66667	C25 - C27	π*	0.46256	43.12
O9	LP(2)	1.86446	C31 - C33	π*	0.02676	12.18
O9	LP(3)	1.64243	C31 - C33	π*	0.02676	14.13
O11	LP(3)	1.67832	C31 - C33	π*	0.02676	34.81
O7 - C24	π*	0.30377	C28 - C30	π*	0.45446	56.43
C18 - C22	π*	0.43457	C15 - C17	π*	0.41651	252.50
C19 - C23	π*	0.44531	C15 - C17	π*	0.41651	175.68
C20 - C26	π*	0.44531	C16 - C21	π*	0.41511	270.61
C25 - C27	π*	0.46256	C16 - C21	π*	0.41511	164.47
C31 - C33	π*	0.02676	C28 - C30	π*	0.45446	234.33

4. DISCUSSION and CONCLUSION

Among metal nanoparticles, AgNPs have attracted great interest for many years due to their unique optical (SPR), electrical and physicochemical properties. They can be synthesized easily and can be obtained as monodisperse in a narrow particle size range. The synthesis of AgNPs by the green synthesis method, which is carried out using various biological sources such as plants and bacteria, draws attention to the fact that toxic chemicals are not used and it is an environmentally friendly method. First, the polphenols in the ethanol/water extract of propolis were determined and the synthesis of silver nanoparticles was successfully carried out. It has been found by theoretical studies that Epigallocatechin gallate, Kaempferol, and Quercetin compounds in propolis are effective in the synthesis of silver nanoparticles.

Since the energy difference between the orbitals of the molecules formed by the interaction of Epigallocatechin gallate, Apigenin, Gallic acid, Kaempferol, and Quercetin molecules with Ag^{+1} is quite low, they are more reactive and soft molecules than other molecules.

As a result, it was calculated that phenolic molecules Epigallocatechin gallate, Kaempferol, and Quercetin with high NLO values bind better with Ag^{+1}. Therefore, these compounds are effective in obtaining nano-Ag. At the same time, Epigallocatechin gallate, Kaempferol, and Quercetin phenolics with high NLO properties are thought to be worth examining as organic optical technological materials.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Aslı Ozturk Kiraz: Investigation, resources, visualization, theoretical calculation, formal analysis, and writing - original draft. **Mine Sulak:** Methodology, supervision, validation and analyses to be performed. **Yesim Kara:** Investigation, resources and preparation of propolis extract. **Izzet Kara:** Methodology, supervision, theoretical calculation, formal analysis.

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Research Article

Antimicrobial potential of lemon and onion extracts against gram-positive and -negative bacteria

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Abstract: Antimicrobial potentials have been widely analyzed with different sources; however, plant-based antimicrobial compounds are greatly welcome due to their greener characteristics. This study revealed the importance of antimicrobial compounds from the herbal extracts of lemon and onion. The extracts were tested against gram-negative (*Escherichia coli*) and gram-positive (*Bacillus subtilis*) bacteria. Disc-diffusion and well-diffusion on an agar plate and tributary methods were followed to demonstrate the antimicrobial potentials of the above herbal extracts. Further, different volumes of ampicillin at the concentration of 1 mg/ml were used to compare the genuine bacterial inhibition (3 µL with 1.5 cm zone). Lemon behaved excellently in a way by displaying better bacterial inhibition against both *E. coli* (3 µL with 1.2 cm zone) and *B. subtilis* (3 µL with 0.6 cm zone), whereas onion extract was not at the level of lemon extract; however, it still displayed a good inhibition. The turbidity assay confirms the inhibition efficiency of lemon and onion against both *E. coli* and *B. subtilis.* In the liquid medium lemon shows higher inhibition (2 & 3 folds) on bacteria than that of ampicillin and onion. Cell count and UV-vis spectroscopy analysis at 600 nm also conform to the efficacy of lemon inhibition against *E. coli* and *B. subtilis.* This experiment confirms that lemon extract is an excellent and better substitute for commercially available ampicillin for bacterial inhibition.

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1. INTRODUCTION

Phytochemicals are compounds originally from plants, predominantly generated as secondary metabolites (Idehen *et al*., 2017; Choudhari *et al*., 2020). The secondary metabolites have been identified as terpenes, phenolic, and nitrogen compounds. For a long period, plant-based extracts were used to produce drugs against a range of diseases, called "traditional medicine". Most of the traditional medicines are considered to be "broad-spectrum" and found to have antimicrobial activities against a wide range of strains (Jamshidi-Kia *et al*., 2018; Osungunna 2020; Vaou *et al*., 2021). However, microbial disease transmission, especially bacteria show the genetic potential to acquire and transmit resistance to drugs. Identifying the natural compounds has antimicrobial activity as therapeutic agents are mandatory for medicinal purposes. The antimicrobial activity of natural products is beneficial, and they do not show side

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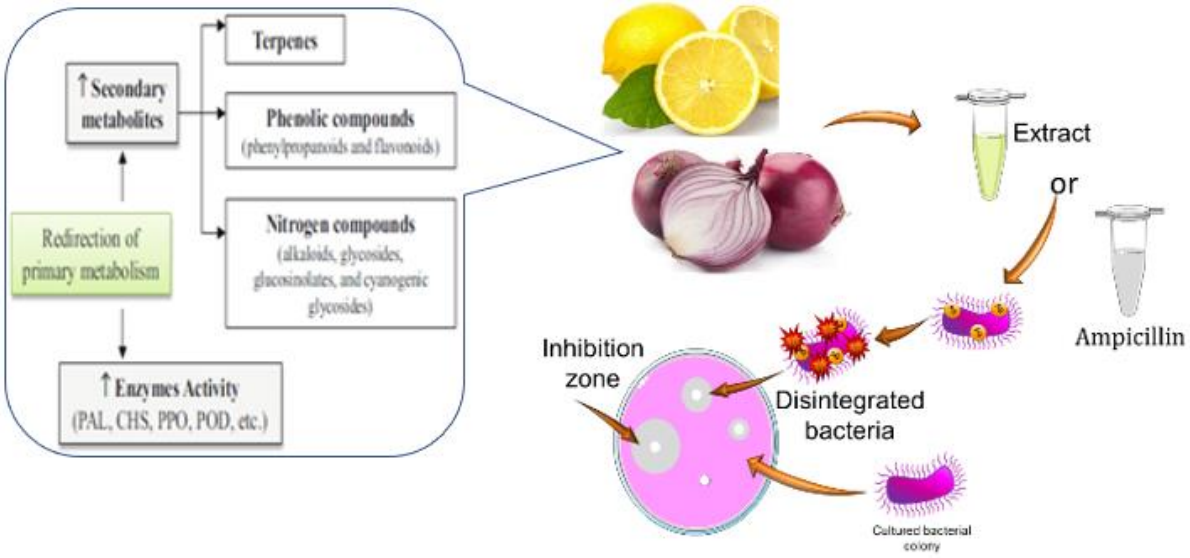
effects (Anyanwu & Okoye 2017). Among different plant species, citrus fruits are proven to have antifungal, antibacterial, antiviral, and anticancer activities (Kadhim Hindi & Ghani Chabuck 2013; Oikeh *et al*., 2016). Lemon (*Citrus limon*) is a commonly available inexpensive fruit that belongs to the Rutaceae family, popular for its medicinal and culinary uses. Lemon contains 5% of citric acid, and exhibits a higher level of vitamin C. Similarly, Onion (*Allium cepa*) is the oldest plant, containing minerals and vitamins. It is mainly used for culinary as well as medicinal purposes (Houba & Adam 1964; Ghaffariyan *et al*., 2012; Safaei-Chaeikar & Rahimi 2017). In this research, the antimicrobial activity of lemon and onion extracts was compared with the commercial antibiotic Ampicillin against bacterial pathogens. Ampicillin is the penicillin type of antibiotic and is used to treat several diseases caused by bacterial infections, including gonorrhoea, pneumonia, and salmonella. Even though it is effective against bacteria, it causes the side effects such as rash, fever, hives, vomiting, anaemia, nausea, headache, high count of white blood cells, and allergic reaction. So, it is mandatory to identify a suitable substitute for ampicillin with natural products to avoid the side effect. Herein, to reveal the plant-based antimicrobial compounds, lemon and onion extracts are tested against gram-negative and -positive bacterial species (Figure 1).

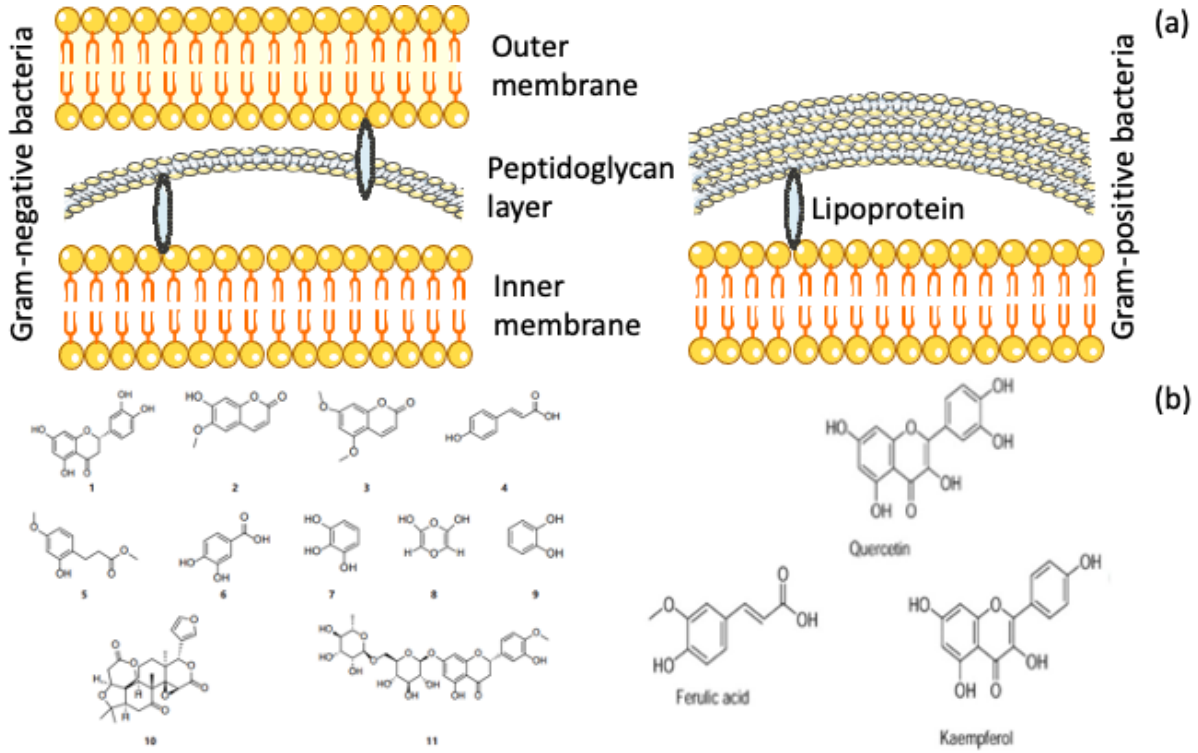
Figure 1. Primary metabolism of the plant. It results in increased synthesis of secondary metabolites. The secondary metabolites are metabolically classified into three main groups, terpenes, phenolic compounds, and nitrogen compounds. The steps involved in extraction and the mode of inhibition are displayed.



Two bacterial species namely *Escherichia coli* (*E. coli)* and *Bacillus subtilis (B. subtilis)* were evaluated for antimicrobial activity. These bacterial species belong to the class gram-negative and gram-positive bacteria, respectively. This discrimination is based on the gram-stain retaining capability of these species. Usually, gram-negative bacteria have a thin peptide-glycan layer and wash out the stain during the test. Whereas gram-positive bacteria have a thick peptidoglycan layer and retain the stain on the cell wall even after severe washing during the staining procedure (Figure 2a). The primary issue of microbial species is resistance to antimicrobial drugs, and it is considered one of the current challenges faced for long. However, researchers are continuously working on plant-based antimicrobial compounds as they are attractive and very often devoid of the side effects found to associate with synthetic antimicrobial compounds (Figure 2b). For the current study, two commonly occurring bacterial species (*E. coli* and *B. subtilis*] were considered to test the antimicrobial compounds from the edible plant sources. Strains of *E. coli* cause the illness of fever, diarrhea, vomiting, and

abdominal pain called an “opportunistic pathogen”. *B. subtilis* is widely spread in the open environment and causes food poison, called a “food pathogen”. Food-borne illnesses are an increasing global public health concern that necessitates more effective preservation techniques. Citric acid, ascorbic acid, minerals, flavonoids, and essential oils are just a few of the bioactive substances included in lemons. Similarly, the onion plant has a higher potential for antimicrobial compounds. To get more insight, the above bacterial survivals were analyzed by natural products of lemon and onion and compared with the established antibiotic “Ampicillin”.

Figure 2. Secondary metabolites and membrane structures. (a) Structure of the phenolic compounds in lemon. Eriodictyol (1), scopoletin (2), citropten (3), p-coumaric acid (4), 2-hydroxy4-methoxy benzene-propanoic acid methyl ester (5), protocatechuic acid (6), pyrogallol (7), diglycolic anhydride (8), catechol (9), limonin (10), hesperidin (11). (b) The difference in cell wall pattern from the bacteria. Gram-negative bacteria have thin peptidoglycan and gram-positive bacteria have thick peptidoglycan.



1.1. Mechanism of Antimicrobial Activity of Plant Extract Against Bacteria

Bioactive compounds such phenolics, flavonoids, ascorbic acid, vitamins, and essential oils are known to be present in citrus fruits. These compounds are believed to be the cause of a number of health advantages, including antibacterial, anti-inflammatory, anticancer, and antioxidant effects. The ability of phenolic compounds to act as electron donors in free radical reactions is commonly associated with antioxidant activity (Irkin *et al*., 2015; Hojjati & Barzegar, 2018). The main phenolic compounds of lemon are eriodictyol, scopolamine, citropten, protocatechuic acid, catechol, limonin, hesperidin, and diglycolic anhydride. Due to the antioxidant activity of phenolic compounds, lemon has been used for various purposes such as the formulation of healthy food, pharmaceutical and cosmetic products (M’hiri *et al*., 2017; Saifullah *et al*., 2019; Shaygannia *et al*., 2021). Figure 2b shows the structure of the phenolic compounds found in lemon. These phenolic compounds have the propionic side chain, which changes its behaviour as less polar than the protocatechuic acid (hydroxybenzoic acid). Considering the cell membrane permeability, caffeic acid is less polar, exhibits lipophilicity, and interferes with the permeability. Andrade *et al*., (2014) have proved that presence of α-tocopherol, lipophilic

compound damages the phospholipid and proteins in the membrane and affects these molecules by playing a pivotal role to increase membrane permeability. The catechin research has also proven that the number of alkyl chain carbons increases the antibacterial properties of these substances. This property facilitates the transport through the cell membrane and is related to the stronger antibacterial potential (Kępa *et al*., 2018).

Similarly, onions also contain high levels of phenolic compounds. The major phenolic compounds in onions are ferulic acid, gallic acid, kaempferol, quercetin, and chlorogenic acid (Figure 2b). Among these, gallic acid is at a higher rate and quercetin plays a major role with functional benefits such as anticancer, antivirus, antihistamine, and anti-inflammatory (Liguori *et al*., 2017). In addition, as stated above thicknesses in the bacterial membrane influences the penetration of these compounds, ultimately influencing the antibacterial action. It is hard to pinpoint the exact mechanism of molecular diffusion in the membrane, however, it is commonly agreed that the compounds penetrate through the periplasmic space of the cell wall and move into the cytoplasm, ultimately causing "pits" formation to the inner portion of the cell membrane. With this action, there is an enhancement in the cell permeability and causes more to be diffused, leading to the malfunction of the organism (Ramanathan & Gopinath 2017).

This research is aimed to compare the antimicrobial activity of extracts from lemon and onion with the commercial antibiotic "Ampicillin", to be tested against the bacterial strains *E. coli* and *B. subtilis.* Since the phenolic compounds and other secondary metabolites from the plant extract show excellent antimicrobial activity, we hypothesized to obtain good antimicrobial activities from the edible plants, lemon, and onion. It is expected that the experiments with both lemon and onion show a greater antimicrobial activity against the bacterial species and be better/comparable to ampicillin.

2. MATERIAL and METHODS

Agar, sterile water (H_2O), Ethanol, and Ampicillin were obtained from Sigma-Aldrich, USA. Hemocytometer (Thermo Fisher Scientific, USA) was used to count the bacteria. Lemon juice was prepared by directly squeezing using the bought fresh lemon from the local market (Malaysia). Onions were washed thoroughly with water and ethanol. And then juice was extracted from the juicer.

2.1. Hemacytometer: Bacterial Cell Counting

Cells are suspended in fluid and a small volume (~10 µL) of the fluid was placed into a special chamber. The hemacytometer was initially cleaned thoroughly using ethanol. The middle chamber with grid was loaded with 10 µL of the overnight culture and covered by the cover glass. The above set-up was placed under the microscope and counted with 5 different squares (with 16 internal squares). The measured counts were averaged for further experiments. The extraction procedure with lemon (*Citrus limon*) and onion (*Allium cepa*) was performed using mortar and pestle. The collected lemon and onion were washed thoroughly, and the pieces of sample were wetted during the grinding process.

2.2. Preparation of Agar-Nutrient Plates

The test microorganisms were cultured on nutrient agar (*Escherichia coli* and *Bacillus subtilis*). About 500 mL of water was added to 7 g of powdered agar-nutrient growth media, which were then autoclaved at 121 °C (15 min). To prevent the agar from setting, autoclaved media was cooled to 60 °C and aseptically placed into 20 cm Petri plates while maintaining sterility. Until they were utilized again, the unused plates were stored at 4°C.

2.3. Disc Diffusion Assay

Active cultures were prepared by transferring a single colony into the autoclaved culture media (5 ml of nutrient broth) and incubated (at 37 °C) for 24 h. The desired cultured bacterial count (10^6 cells) was spread independently on the agar plate. To measure the minimum inhibitory, different dilutions of lemon (as extracted) or onion (as extracted), or ampicillin (from 1 mg/mL) were wetted (with uniform final volume adjusted by the sterile water) a 5 mm-diameter paper disc that was placed on a plate of bacteria-containing agar and left to incubate there overnight. The inhibition areas formed were measured using the conventional ruler.

2.4. Well-Diffusion Assay

Active cultures were prepared by transferring a single colony into the autoclaved culture media (5 ml of nutrient broth) and incubated (at 37 °C) for 24 h. The desired cultured bacterial count (10^6 cells) was spread independently on the agar plate. Using the sterile cork-borer the uniform wells were made and poured with different dilutions of lemon (100% as extracted) or onion (100% as extracted) or ampicillin (from 1 mg/mL) and the uniform final volume was adjusted by the sterile water, then incubated overnight. The inhibition areas formed were measured using the conventional ruler.

2.5. Inhibitory Effect-Dose Dependent Analysis: Turbidity Assay

Active cultures were prepared by transferring a single colony into the autoclaved culture media (5 ml of nutrient broth) and incubated (at 37 °C) for 24 h. The desired cultured bacterial count (10^6 cells) was re-inoculated into each sterile culture tube containing 3 ml of culture medium. To the different tubes, varied amounts of lemon/onion extracts were added with equal final volumes by adjusting with sterile water. Kept all the culture tubes at 37° C incubator shaker overnight. The next day the cultures were measured for bacterial density using a UV-Visible spectrophotometer at 600 nm.

3. RESULTS

In this study, the extracts from edible parts of the plants (lemon and onion) were collected and tested for their antibacterial activities against *E. coli* and *B. subtilis*, and ampicillin was tested as a positive control. The experiment is designed to check the antimicrobial potential using the cultures in a liquid medium and on a solid agar surface. It is expected that depending on the diffusion and conjugation of the inhibitory compounds from the lemon/onion to the microbial cell wall, and the result will vary. Further, the antibacterial potential will differ from species to species. To make clear on this the selected test bacterial species are from gram-negative and -positive classes, originally categorized based on the gram-staining. Both gram-negative and -positive strains have different thicknesses of the peptidoglycan layers thin and thicker, respectively. This will make differences in the antibacterial activities due to dissimilar rates of penetration. In addition, the compounds from lemon and onion will display differences in inhibition due to the variations among the secondary metabolites extracted from the lemon and anion. To obtain solid results, both disc- and well-diffusion methods were followed and compared. Using a similar number of cells with the aid of a hemacytometer, different experiments were performed, and they yielded a higher confident output.

3.1. Diffusion Assay for Identifying the Antimicrobial Activity of Lemon and Onion Extract on *E. coli* and *B. subtilis*

Figure 3 shows the disc diffusion and well diffusion assay for Ampicillin, lemon, and onion against *E. coli*. As shown in Figure 3a, four different volumes (3.125, 6.25, 12.5, and 25 µL) with fixed concentration of Ampicillin, lemon, and onion extracts were tested against *E. coli*. It was noted that with increasing the volumes of Ampicillin, lemon, and onion, the diffusion was increased. At 3.125 µL, Ampicillin shows the highest diffusion, with increasing the

concentration to 6.25 µL, onion and lemon show the similar levels of diffusion (Table 1). In the good diffusion assay, lemon is more predominant than ampicillin and onion. Onion shows less diffusion than others. In the case of lemon, all four concentrations showed the highest rate of diffusion (Figure 3b). The diffusion rate of onion and lemon was close to ampicillin and in particular, lemon is closer to commercial Ampicillin (Figure 4a). It was noted that lemon extract works better than ampicillin and onion against *E. coli* (Figure 4a, b, Table 2).

Figure 3. (a) Results obtained by the disc-diffusion assay. (b) Results obtained by the disc-diffusion assay. Results for all three test samples were displayed against *E. coli*.

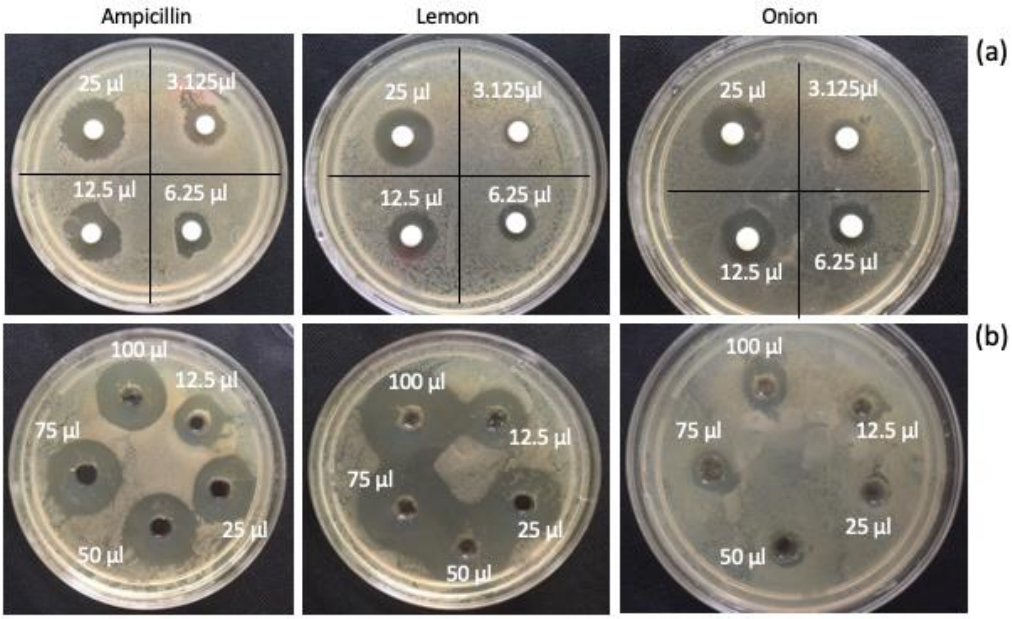


Figure 4. (a) Results obtained by the disc-diffusion assay. (b) Results obtained by the disc-diffusion assay. Results for all three test samples were displayed against *B. subtilis*.

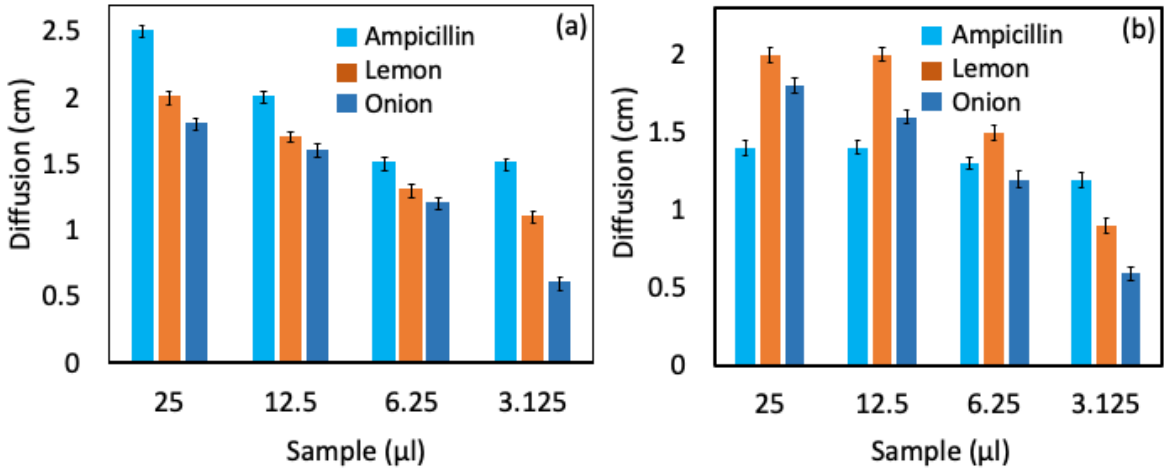


Table 1. Qualitative and quantitative assessments on disc diffusion assay.

Sample	Qualitative data		Quantitative data	
	E. coli	*B. subtilis*	*E. coli*	*B. subtilis*
Ampicillin	Smaller diffusion at 3 and 6 µL, larger diffusion at 12 and 25 µL	Smaller diffusion at 3 and 6 µL, larger diffusion at 12 and 25 µL	3 µL-1.5 cm 6 µL -1.5 cm 12 µL-2 cm 25 µL-2.5 cm	3 µL-1.2 cm 6 µL -1.3 cm 12 µL-1.4 cm 25 µL-1.4 cm
Lemon	With increasing the concentration, gradual increments in the inhibition	With increasing the concentration, gradual increments in the inhibition	3 µL-1.2 cm 6 µL -1.3 cm 12 µL-1.4 cm 25 µL-1.4 cm	3 µL-0.9 cm 6 µL -1.5 cm 12 µL-2 cm 25 µL-2.1 cm
Onion	With increasing the concentration, gradual increments in the inhibition	Diffusion depends on the concentration, but not clear enough.	3 µL-0.6 cm 6 µL -1.2 cm 12 µL-1.6 cm 25 µL-1.8 cm	3 µL-0.5 cm 6 µL -1.2 cm 12 µL-1.6 cm 25 µL-1.8 cm

Table 2. Qualitative and quantitative assessments of well diffusion assay.

	Qualitative data		Quantitative data	
	E. coli	*B. subtilis*	*E. coli*	*B. subtilis*
Ampicillin	With increasing the concentration, gradual increments in the inhibition	With increasing the concentration, gradual increments in the inhibition	12 µL-1.6 cm 25 µL -2 cm 50 µL-2.3 cm 75 µL-2.3 cm 75 µL-2.4 cm	12 µL-1.5 cm 25 µL -1.7 cm 50 µL-2 cm 75 µL-2 cm 75 µL-2 cm
Lemon	With increasing the concentration, gradual increments in the inhibition	With increasing the concentration, gradual increments in the inhibition	12 µL-1.7 cm 25 µL -2 cm 50 µL-2.7 cm 75 µL-2.7 cm 75 µL-3 cm	12 µL-2 cm 25 µL -2.5 cm 50 µL-2.8 cm 75 µL-3 cm 75 µL-3.4 cm
Onion	Small diffusion was noticed	Small diffusion was noticed	12 µL-1.1 cm 25 µL -1.1 cm 50 µL-1.1 cm 75 µL-1.2 cm 75 µL-1.3 cm	12 µL-0.8 cm 25 µL -0.8 cm 50 µL-1 cm 75 µL-1.2 cm 75 µL-1.3 cm

Similar experiments were conducted with the gram-positive bacteria *B. subtilis***.** Figure 5 shows the disc diffusion and well diffusion assays for Ampicillin, lemon, and onion against *B. subtilis***.** As shown in Figure 5a, b, four different volumes (3.125, 6.25, 12.5, and 25 µL) with fixed concentrations of Ampicillin, lemon, and onion were tested against *B. subtilis***.** In this case, lemon works well in both disc diffusion and well diffusion assay for all concentrations against *B. subtilis***.** It was visibly observed with lemon, the diffusion was spread widely in all the areas of the plate and is greater than the plate with Ampicillin. The length of diffusion was higher with lemon followed by ampicillin and onion in both disc diffusion and well diffusion assay (Figure 6a, b). From this result, it was concluded that lemon and onion work well against *E. coli* and *B. subtilis*. In particular, lemon works better than commercially available Ampicillin.

Figure 5. Disc diffusion assay. (a) *E. coli*; (b) *B. subtilis*. Inhibition area (cm) by ampicillin, lemon, and onion are shown. Data are averaged with triplicates and indicated by error values.

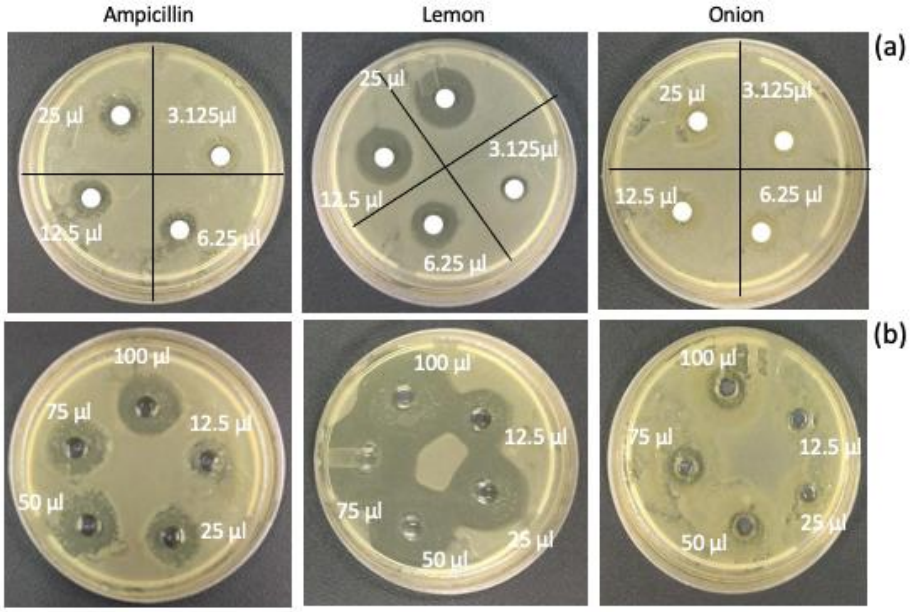
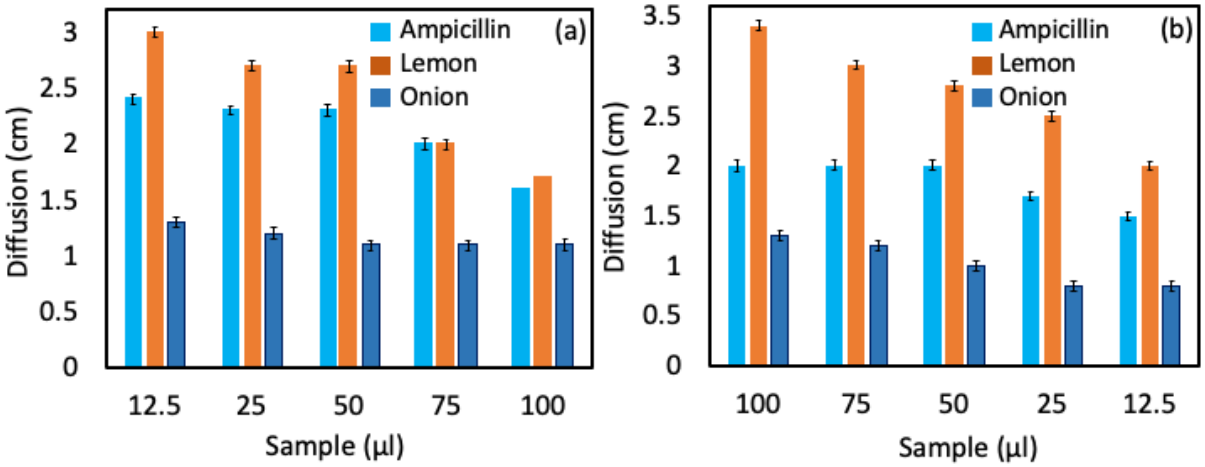


Figure 6. Well diffusion assay. (a) *E. coli*; (b) *B. subtilis*. Inhibition area (cm) by ampicillin, lemon, and onion are shown. Data are averaged with triplicates and indicated by error values.



3.2. Turbidity Assay for Identifying the Antimicrobial Activity of Lemon and Onion Extract on *E. coli and B. subtilis*

Since lemon and onion suppress the growth of *E. coli* and *B. subtilis*, further results were confirmed with the liquid medium by turbidity assay. In the liquid medium, the bacteria were grown and treated with lemon, onion, and ampicillin, and the results were compared. As shown in Figure 7, lemon shows an equal level of inhibition with commercially available ampicillin in both *E. coli* and *B. subtilis*. Onion inhibits the growth of bacteria at higher concentrations. The samples are further analyzed with UV-Vis spectroscopy. The optical density of the solution was measured. The liquid treated with lemon and ampicillin shows a lower O.D. compared with inion (Table 3). Lemon shows lower OD at higher concentrations compared with ampicillin. This result confirmed that lemon works well than ampicillin in a liquid medium compare with a solid place. For further confirmation the changes were validated by cell counting and measurement on biomass.

Figure 7. Turbidity assay for qualitative assessment. +++: Good growth; ++ Moderate growth; + Less growth; - Not significant.

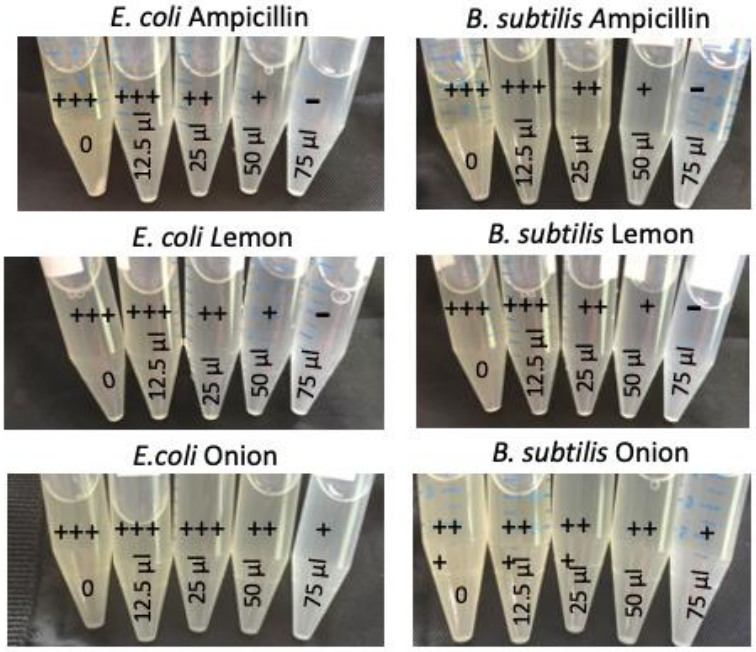


Table 3. Inhibitory Effect-dose dependent analysis (after 24 hrs; Turbidity).

Sample volume	Ampicillin (O.D)		Lemon (O.D)		Onion (O.D)	
	E.coli	*B. subtilis*	*E. coli*	*B. subtilis*	*E. coli*	*B. subtilis*
0	0.9	0.8	0.9	0.8	0.9	0.8
12.5 µL	0.85	0.74	0.78	0.72	0.86	0.77
25 µL	0.72	0.69	0.62	0.68	0.79	0.7
50 µL	0.61	0.57	0.54	0.59	0.71	0.59
75 µL	0.4	0.5	0.33	0.48	0.65	0.53
100 µL	0.2	0.4	0.11	0.3	0.3	0.43

3.3. Cell Count and Biomass of the Bacteria in the Liquid Medium

For further confirmation of the effect of lemon and onion effect against the bacteria, cell count and biomass were calculated. The following are the basic calculation made with the hemacytometer measurements.

The number of cells/ cubic mm = Number of cells counted/ mm^2 X dilution X 10

The number of cells/mm =Number of cells counted / mm^2 X dilution X 10 000

As shown in Figure 8a, cell count was decreased when increasing the concentration of lemon, onion, and ampicillin. Lemon shows the lowest level of cell count compared with ampicillin and lemon (Tables 4 & 5). Similarly, the Inhibitory effect-bacterial biomass was calculated for further confirmation. The cultures were filtered on a pre-weighed Whatman filter paper. The collected biomass was dried in an oven at 80°C. After drying the final weight was calculated by subtracting the initial weight of the filter. Figure 8b shows the biomass of the bacteria from the liquid treated with lemon, onion, and ampicillin. In both of the bacteria, lemon shows a lower mass of bacteria, which confirms the strong inhibition of lemon against bacteria compared with ampicillin and lemon.

Figure 8. Quantitative analysis with bacterial growth. Cell count (a) and Biomass (b) analyses are shown.

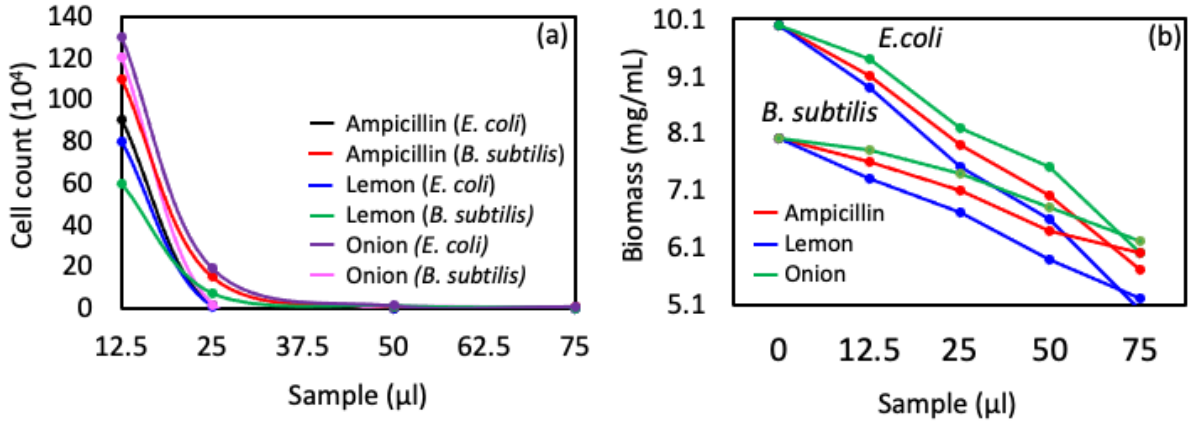


Table 4. Biomass & Cell count-Dose dependent analysis (after 24 hrs: *E. coli*).

Sample volume (µL)	Ampicillin		Lemon		Onion	
	Biomass (mg/ml)	Cell count	Biomass (mg/ml)	Cell count	Biomass (mg/ml)	Cell count
0	10	18 x 10^6	10	18 x 10^6	10	18 x 10^6
12.5	9.1	0.9 x 10^5	8.9	0.8 x 10^5	9.4	12 x 10^5
25	7.9	1.1 x 10^4	7.5	0.91 x 10^4	8.2	1.7 x 10^4
50	7.0	0.2 x 10^4	6.6	0.6 x 10^3	7.5	0.8 x 10^4
75	5.7	0.7 x 10^3	5.0	0.1 x 10^3	6.0	0.1 x 10^4

Table 5. Biomass & Cell Count-Dose dependent analysis (after 24 hrs: *B. subtilis*).

Sample volume (µL)	Ampicillin		Lemon		Onion	
	Biomass (mg/ml)	Cell count	Biomass (mg/ml)	Cell count	Biomass (mg/ml)	Cell count
0	8	16 x 10^5	8	16 x 10^5	8	16 x 10^5
12.5	7.6	11 x 10^5	7.3	6 x 10^5	7.8	13 x 10^5
25	7.1	1.5x 10^5	6.7	1x 10^4	7.4	1.9x 10^5
50	6.4	1 x 10^4	5.9	0.9 x 10^3	6.8	1.2 x 10^4
75	6.0	0.5 x 10^4	5.2	0.4 x 10^3	6.2	0.9 x 10^4

4. CONCLUSION

Research in the medical field is necessary to improve human health and to increase human life span. Different research is going on about threatening diseases including the diseases caused by bacteria and viruses. There are different ways to treat microbial infections; however, the currently available medicines cause side effects. Natural medicine is better because it can avoid side effects; particularly herbal plants can cure microbial infections. Towards this direction, this study has been launched to make the expansion for the potential applications of the plant extracts to be used as antibacterial agents. For this analysis, two common edible plants, namely lemon and onion have been chosen for the gram- and gram-positive bacteria, namely *E. coli* and *B. subtilis*, respectively. The following conclusions have been made based on the obtained results. With the disc-diffusion assay the commercial antibiotic, ampicillin, lemon, and onion can inhibit the growth of *E. coli* and *B. subtilis*. In the case of *E. coli*, ampicillin shows the highest inhibition followed by lemon and onion. Interestingly in a well diffusion assay lemon has higher antibacterial activity against *E. coli* and *B. subtilis*. Followed by lemon extract, ampicillin displays better antibacterial activity against *B. subtilis*, whereas onion shows the least activity. The biomass obtained from the liquid medium inhibition assays support the results by showing a reduction in the biomass compared to the control test in the absence of additional compounds. Cell counts performed with the same assays show the decrement in the cell count with increasing the plant extract or ampicillin concentration. Considering the limitation with the above methods, inclusion of filtration step with lemon or onion extract makes changes in the antimicrobial activity due to the attachment of important compounds on the filters. In addition, when go for a large scale needs a fine optimization, and the above methods work also well with different sources of plant material.

Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

Authorship Contribution Statement

Nagomi Gopinath: Investigation, Resources, Visualization, Software, Formal Analysis, Methodology, Supervision, and Validation. and Writing -original draft.

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